MORPHOMETRIC ANALYSIS OF CD1a POSITIVE LANGERHANS CELLS IN THE HUMAN TYMPANIC MEMBRANE

Dissertation submitted in partial fulfillment of the Degree of Master of Surgery (Anatomy) of the Tamil Nadu Dr. M.G.R. Medical University, Chennai

by

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CERTIFICATION

This is to certify that the dissertation entitled *"Morphometric analysis of CD1a positive Langerhans cells in the human tympanic membrane"* is based on the results of the work carried out by

Dr. TRIPTI MERIEL JACOB

for the degree of Master of Surgery under my supervision. The work reported in this dissertation has not been submitted to any other university for the award of a degree.

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CONTENTS

INTRODUCTION

1. INTRODUCTION

The immune system consists of lymphoid organs and a heterogeneous group of motile cell types that are involved in the defense of the body against invasion by bacteria, viruses and other foreign bodies.[1]

Dendritic cells form a system of antigen presenting cells that is widely distributed in the body. They constitute trace population in lymphoid and non-lymphoid tissues and in the circulation. They are characterized by their typical dendritic and “veiled” morphology, by their constitutive of high levels of major histocompatiblity complex class II molecules on their surface, and their outstanding capacity to initiate primary immune responses.

Dendritic cells occur in two states of differentiation. In the immature state they are highly specialized for processing foreign protein antigens; in the mature state, they efficiently stimulate resting antigen- specific T cells.

Dendritic cells can migrate from the non-lymphoid tissues, where they reside in immature state, via the afferent lymphatics or blood to the T cell dependent areas of lymphoid organs like lymph nodes and the spleen. There, they appear as mature dendritic cells.

Therefore dendritic cells are ideally suited to mediate important aspects of immunogenicity: they can acquire antigens in the tissues and process them in an immunogenic form; they can carry that immunogens to the lymphoid organs; and

they can find and efficiently activate antigen-specific T cell clones and thus generate an immune response.[2]

Langerhans cells have been well documented in the epidermis of skin and are considered to be the sentinel cells of the cutaneous immune system. Innumerable studies have been done describing their number, morphology and distribution in the skin, but the knowledge regarding their role in the tympanic membrane is very limited.

There has been some controversy regarding the presence and distribution of Langerhans cells in the normal human tympanic membrane. They have been reported in the tympanic membranes of patients with chronic otitis media, especially in those with aural cholesteatoma. However, the increase in the number of Langerhans cells in these conditions has not been quantified.

A standard technique of identifying the Langerhans cell has been shown to be by immunohistochemical testing for the cell surface antigen CD1a.

This study proposes a morphometric analysis, looking at the number and morphology of Langerhans cells in the stratified squamous epithelium and sub-epithelial connective tissue of normal human tympanic membranes, as well as tympanic membranes from individuals with chronic ear infection by immunohistochemical testing for the cell surface antigen CD1a, and thereby establishing their role in the immunology of the disease.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES OF THE STUDY

Aim: To quantitatively analyze, and to study the distribution and morphology of CD1a positive Langerhans cells in normal cadaveric and diseased human tympanic membrane.

Objectives:

1. To confirm the presence of CD1a positive Langerhans cells in normal human tympanic membrane.

2. To study the distribution and morphology of CD1a positive Langerhans cells in normal human tympanic membrane.

3. To study the distribution and morphology of CD1a positive Langerhans cells in tympanic membranes of patients with chronic otitis media of both the tubotympanic and atticoantral varieties.

4. To quantify the number of CD1a positive Langerhans cells in normal versus diseased tympanic membrane.

REVIEW OF LITERATURE

3. REVIEW OF LITERATURE:

3.1: DENDRITIC CELLS:

Immunity is the ability of the body to defend itself against invading agents such as bacteria, toxins, viruses and foreign tissues. At the core of the immune system is the ability to distinguish “self” and “non self”.

Classically, the immune response has been differentiated into innate immunity and acquired immunity. In general, immunity consists of two closely allied components. One consists of the formation of specially sensitized lymphocytes that have the capacity to attach to the foreign agent and destroy it. This is called **cellular** or **cell mediated immunity**. The other consists of circulating antibodies produced by the body that are capable of neutralizing, eliminating or rendering ineffective an invading agent. This is called **humoral** or **antibody mediated immunity**.

Unlike B cells, T cells can only detect foreign substances in specific contexts. In particular, T lymphoytes will recognize a foreign protein only if it is first cleaved into small peptides, which are then displayed on the surface of a second host cell called an **antigen presenting cell**.[3]

The macrophage is the typically described antigen presenting cell. However, there is one function where it is deficient, namely, the priming of naive lymphocytes. It is believed that cells other than macrophages prime T helper cells and these are the **dendritic cells**.[1]

Dendritic cells are a system of antigen presenting cells that function to initiate several immune responses such as the sensitization of MHC-restricted T cells, the rejection of organ transplants, and the formation of T-dependent antibodies.[4] They are so named because of their long dendritic processes that can be visualized with special stains under light microscopy.[5, 6]

It is the wide distribution of dendritic cells in vivo that renders them especially suited to capture antigens, and subsequently to sample and select T cells with reactivity for that antigen.[7] To state a few examples, dendritic cells are distributed throughout the suprabasal zone of the epidermis and in other stratified squamous epithelia,[8] they are found in airways, [9-11] gut mucosa[12, 13] and in the interstitial spaces of most other organs other than the parenchyma of the brain.[14, 15] Dendritic cells are also frequently found in afferent lymphatics where they are called “veiled” cells.[16, 17] In addition, both immature and mature dendritic cells are found in the blood.[7, 18, 19] This is due to their unique capacity to migrate from tissues where they have first encountered an antigen, to T cell areas of lymphoid tissues.[20] Dendritic cell migration via the blood provides access to peri-arterial sheaths of T cells in the spleen, while their migration through the afferent lymph vessels provides access to the para-cortical cells of the lymph node. The T cell areas are not static zones. On the contrary, T cells continually re-circulate through these regions. In doing so, they encounter the numerous antigen-major histocompatability complex rich processes of the dendritic cell. This enhances the ability of dendritic cells to select those T cells that are specific for the

antigens being presented. Dendritic cells also home to sites of delayed hypersensitivity where they again closely associate with T cells.[21]

Types of dendritic cells:

Dendritic cells exist in different forms in different tissues. Broadly, they can be classified into four types, namely[3]:

a. **Langerhans cells of the epidermis:** These are situated in the suprabasal portions of the epidermis and have been described in detail later in this document.

b. **Blood dendritic cells:** Human blood contains two subsets of dendritic cells, namely, an immunologically mature type, having powerful immunostimmulatory function and an immunologically immature type. O' Dohertry et al[18, 19] hypothesized that the immature cells are marrow-derived precursors to tissue dendritic cells, such as epidermal Langerhans' cells. It is also stated that the mature cells are derived from tissues where they have been activated by an antigen and are en route to the spleen or lymph nodes to stimulate T-cell responses there. Purified fresh isolates lack the characteristic morphology, phenotype, and immunostimulatory function of dendritic cells. When placed in culture, the cells mature in a manner resembling the cytokine-dependent maturation of epidermal dendritic cells (Langerhans cells). The cells enlarge and exhibit many cell processes, express much higher levels of major

histocompatibility complex class II and a panel of accessory molecules for T cell activation, and become potent stimulators of the mixed leukocyte reaction.

c. **Veiled cells of the lymph:** These constitute the cells migrating via the afferent lymphatics towards lymphoid tissue.

d. **Interdigitating cells of lymphoid organs:** In the thymus, dendritic cells are confined primarily to the medulla where they are responsible for self tolerance.[7] Thymic dendritic cells process self-antigens and mediate the deletion of self-reactive T cells.[22, 23] In the thymic medulla, and in the T cell areas of peripheral lymphoid organs, these dendritic cells have been called interdigitating cells.[17]

Despite their many names, all the different mature members of the dendritic cell family can exhibit similar features.[7]

In contrast, there exists a different type of dendritic cell found in the B cell areas of follicles of lymphoid organs. These are called **follicular dendritic cells** which are large, non-lymphoid cells with elongated cytoplasmic extensions that form the framework of the germinal centre.[24] They are thought to be stromal rather than myeloid cells.[25] They play a central role in events related to humoral immunity in the lymphoid follicle.[26, 27] Follicular dendritic cells function to present

antigens as immune complexes to B cells, rather than processed antigens as MHC-peptide complexes to T cells.

CD1a antigens are present on Langerhans cells and interdigitating cells but not on follicular dendritic cells, reinforcing that Langerhans cells and interdigitating cells are close relatives but despite their name, follicular dendritic cells may be regarded as a different cell type.[28, 29] In addition, follicular dendritic cells do not have the typical Birbeck granules in their cytoplasm which distinguish them from Langerhans cells.[24]

Apart from the stratified squamous epithelium of skin, dendritic cells are also seen scattered through the dermis or subepithelial connective tissue. The origin of these dermal dendritic cells is controversial, as these cells are CD1a positive but lack Birbeck granules which are characteristic of Langerhans cells. Thorbecke et al[30] reported that the dendritic cells found in the subepithelial connective tissue were similar to Langerhans cells, and they stated that CD1a positivity further re-emphasised their similarity. Lenz et al studied dendritic cells cultured from human and murine dermis. They postulated three theories based on these findings regarding the origin of the dermal dendritic cells. a) They may originate from epidermal Langerhans cells or their precursors on their way into the epidermis. b) Alternatively, they may originate from epidermal Langerhans cells on their way from the epidermis to the draining lymphnodes. c) Finally, the majority of dermal dendritic cells may be derived from a resident population of presumably immature dendritic cells in the dermis, analogous to the population of Langerhans cells in the epidermis.[5]

3.2: THE LANGERHANS CELL:

3.2.1: History:

The Langerhans cell of mammalian stratified squamous epithelia has intrigued morphologists for many years. Since their discovery in the skin as a distinct cell population with characteristic cell processes more than a century ago, these cells remained unstudied until a few decades ago.[31]

Epidermal Langerhans cells were first described in 1868 by Paul Langerhans (born Berlin, Germany, 25 July 1847; died Funchal Madeira, 20 July 1888), the son of a well know physician in Berlin, who studied medicine at the Universities of Jena and Berlin, graduating in 1869. He made an outstanding contribution to medicine while still an undergraduate student when he described a new epidermal cell in a paper entitled, “Ueber die Nerven der menschlichen llaut”, [31] which when translated from German reads, “On the nerves of the human skin”. Using gold chloride techniques, he described the dendritic non-pigmentary cells in the epidermis, which he regarded as intraepidermal receptors for extracutaneous signals of the nervous system. These cells were subsequently designated with his name.[32]

In 1959, Silvers[33] first demonstrated that aurophilic dendritic cells of the epidermis were not of neural crest origin and were thus, not related to melanocytes.[34]

Birbeck et al[35] later used electron microscopic techniques to show that Langerhans cells contain a characteristic organelle referred to as the **Birbeck granule** or the **Langerhans cell granule**.

However, the physiological role of these cells remained unknown for a long time.

In 1973, Inga Silberg[36] discovered that the epidermal Langerhans cells represent the most peripheral outpost of the immune system. Only subsequently were they identified as dendritic cells that reside in the epidermis in an immature state, but have the capacity to migrate and develop into mature lymphoid dendritic cells. [32]

3.2.2: Location and distribution:

Langerhans cells are widely distributed in the body. They are situated in the suprabasal portions of the epidermis.[2, 4, 37]

Cell-kinetic studies have established the origin of the Langerhans cell. After allogenic bone marrow transplants, Langerhans cells are seen to be completely replaced by donor cells within a few weeks. This has provided the basis for the concept that Langerhans cells are derived from a mobile pool of bone marrow derived precursors that are constantly recruited to the skin.[38, 39]

Under normal circumstances, the turnover rate is slow. Mitoses of Langerhans cells are rare. However they are probably continually renewed and replaced in the periphery of the body.

They are present in any stratified squamous epithelium, of either ectodermal or endodermal origin, which has the capacity to keratinize.[40]

Bauer et al,[41] in a study of mammary skin using a confocal laser scanning microscope, found that a strikingly constant ratio between Langerhans cells and

other epidermal cells in healthy skin is maintained. Thus, the variation in surface density from area to area can be explained by the varying thickness of the epidermis.

3.2.3: Function of the Langerhans Cell:

The physiological role of Langerhans cells remained unknown for a long time. Detection of Fc-Ig and C3 receptors[42] and Ia antigen[43, 44] established the cell as a form of specialized epidermal macrophage. Recent studies now suggest that these cells act constructively as antigen presenting cells in the immunological reactions of the skin.[45]

As members of the dendritic cell family of antigen presenting cells, Langerhans cells have crucial roles in the initiation of cellular immune responses to environmental antigens.[6, 8, 46, 47] Epidermal Langerhans cells are thought to be sentinel cells of the cutaneous immune surveillance system.[45] Upon activation, they increase their expression of MHC class II.[48] They appear to recognize antigens and present them to T lymphocytes, and possibly macrophages.[49, 50] Evidence suggests that they may produce lymphokines, hydrolytic enzymes and even prostaglandins.[30]

Research by Hunger et al[51] indicates that the function of antigen presentation in Langerhans cells is mediated by the Langerhans cell-specific pattern recognition receptor, langerin, as well as the antigen presenting molecule CD1a, which is highly expressed by these cells.

Langerhans cells and thymus derived T lymphocytes have always had a special affinity for each other. Experimental evidence indicates that Langerhans cells migrate through the afferent lymphatics to the draining lymph nodes,[37, 52] carrying antigenic information on their cell membrane. T-cells will then respond and multiply.[53] By re-circulating through the body, these cells will meet their challengers again in the periphery. Evidences for the fact that these cells are capable of movement are many.[30] Ultrastructurally, they sometimes display networks of microfilaments[54] with dimensions of actin as well as a prominent system of microtubules[55]. These features are usually found in cells that move. Work by Kripeke et al[56] and others[16, 57] showed that following topical sensitization of nude mice at the site of an allogenic graft, the antigen-bearing dendritic cell found within the draining nodes were of graft donor origin, giving a compelling argument that epidermal Langerhans cells are stimulated to migrate via afferent lymphatics to the draining nodes. During this migration, they undergo a process of maturation that allows them to present antigens to naïve T cells. Data from these studies shows that Tumor Necrosis Factor α produced locally by keratinocytes is, possibly, one stimulus for migration from the skin.

Epidermal Langerhans cells resemble macrophages in several remarkable respects,[45, 58] for instance, in the presence of Fc and C3 receptors.[42] In addition to the remarkable similarities in their surface markers, there are also analogous roles for these two cells in the initiation of immune responses.[9] In vitro studies have demonstrated that antigen-pulsed, Langerhans cell enriched epidermal cell preparations can induce proliferative responses in immune T cells that are

comparable in magnitude to those induced by similarly pulsed macrophages.[49] Frequent close apposition of Langerhans cells to lymphocytes has been observed at sites of contact hypersensitivity reactions, as well as increased numbers of Langerhans cells observed in dermal lymphatics and draining lymphnodes in these conditions.[40, 52, 59] These observations have lead to the proposal that Langerhans cells pick up antigens in skin and from there move to draining lymph nodes, where they present the antigen to immunologically relevant cells stimulating the resulting T cell response.[60]

3.2.4: Structure:

In routine histological sections stained with hematoxylin and eosin, Langerhans cells cannot be selectively identified. Enzyme histochemical and electron microscopic studies, however, have demonstrated that these cells are suprabasal dendritic cells within the epidermis, and their branched and tapering processes extend far into the surrounding epithelium.[61] They are characterized by their typical dendritic and veiled morphology and by their expression of major histocompatibility complex II.[37]

Figueroa and Caorsi[62] reported an ultrastructural and morphometric study of the Langerhans cells in the normal human exocervix. They classified the Langerhans cells seen into five types, based on the characteristics of the dendritic processes, as follows:

Type I: Only one, unbranched process

Type II: Only one process which divides into branches

Type III: 2 processes

Type IV: Three or more processes

Type V: Also with 3 or more processes but with several collaterals, these, in turn, branched dichotomously.

On an ultrastructural level, Langerhans cells are described to have a lobulated nucleus and lack desmosomes as well as tonofilments. Their cytoplasm contains a well-developed Golgi apparatus, endoplasmic reticulum, mitochondria, centrioles and lysosomes. Most importantly, they produce characteristic granules called Birbeck granules.[63]

Birbeck granules:

Birbeck and coworkers described a specific organelle by which Langerhans cells can be identified at the submicroscopic level, as distinctive rod-shaped or tennis racket-shaped structures of variable length with a central, periodically striated lamella.[35]

Wolff[63] then went on to describe the fine structure of the Birbeck granule from Langerhans cells in the human skin. These granules exhibit different morphological features in different planes of section. Their most common electron microscopic image is that of a rod-like profile with round ends and a limiting membrane approximately 55-60Å thick. Midway between the limiting membranes is a central lamella composed of electron-opaque particles. Owing to the regular spacing of these particles, the rod-like structures acquire a definite pattern of cross-striation. These profiles are very often continuous with round vesicles and thus resemble tennis rackets. Usually the vesicle is located at one

end of the granule, but may also be seen in its centre, with the rod-like structures projecting from its circumference. The large majority of the Langerhans cell granules are seen in the golgi region, but they can also be seen in other areas of the cytoplasm and in the dendritic processes. Not infrequently, the granules are attached to the plasma membrane and their interior is continuous with the extracellular space.

Conflicting theories exist regarding the derivation and function of these granules.[64] The secretion or exocytosis theory suggests they have an intracellular origin from either the Golgi apparatus or endosomes.[63, 65, 66]

Alternatively, the endocytosis theory suggests that Birbeck granules originate from the cell membrane, during receptor-mediated endocytosis, as a prolongation of coated pits that pinch off to form intracellular Birbeck granules.[67,68]

3.2.5: Methods available for identification of Langerhans cells:

Routine histological staining techniques do not visualize the Langerhans cell. However, newer methods of identification have improved this picture.

A definitive technique of identification of these cells would be by identifying the Birbeck granules on an ultrastructural level.[35] This however, requires electron microscopy which is not always a practical option.

Some techniques that have been useful in visualization under light microscopy include staining with Gold chloride,[31] ATPase staining[69, 70] and the L-dopa fluorescence method.[71] Juhlin and Shelley described a technique of

staining with a combined stain of adenosine triphosphate and gold.[59] These techniques present problems with the specificity of reaction and complication of procedure.

Another useful technique is staining with zinc iodide-osmium tetroxide.[72] However, apart from dendritic cells, this has been shown to stain nervous tissue and fat.

Interest in the role of epidermal Langerhans cells in cutaneous immune reactions was stimulated in 1977 by the discovery that these cells express surface markers characteristic of cells of the macrophage-monocyte lineage.[42-44, 56] A number of antigenic markers have been identified on the cell surface of Langerhans cells, some more specific than others. These are S-100 protein,[73, 74] HLA-DR, CD1a,[30, 34, 38, 43, 44, 59, 75, 76] Langerin,[77] E-Cadherin,[78] etc.

Of these, S100 protein is less specific for Langerhans cells than CD1a because it is also present in Schwann cells, fat cells, chondrocytes, interstitial cells of the pineal gland, stellate cells of the adenohypophysis, satellite cells of the adrenal medulla etc.[74, 79] Studies by Palva and Taskinen[59] and Harrist et al[80] showed that CD1a is a better marker for Langerhans cells than HLA-DR, as not all Langerhans cells are positive for HLA-DR antigens. Another drawback of HLA-DR labeling is that other inflammatory cells that may infiltrate the epidermis, such as B cells, some activated T cells and mononuclear phagocytes, also express the HLA-DR antigen.

Thus the most sensitive markers for Langerhans cells are believed to be CD1a and Langerin.[64] Notably, Langerhans cells are the only epidermal cells to

constitutively express MHC class II molecules,[43, 44] CD1a molecules[75] and Langerin[81] at their cell surface.

CD1a staining:

Where a cluster of monoclonals are found to react with the same polypeptide, they clearly represent a series of reagents defining a given marker and are labeled with a CD (cluster of differentiation) number. Currently there are nearly 340 CD numbers assigned, with some of them having subdivisions. The CD1 group presents glycolipid and other non-pepitde antigens to T cells.[1] Human CD1 antigens are a family of structurally related glycoproteins that are non-covalently associated with β_2 microglobulin on their cell surface like MHC class I molecules.[82-84] They are encoded by a set of genes on chromosome 1 in humans. They are involved in the presentation of antigens to T cells.

At least 5 different CD1 genes have been identified and sequenced,[85] of which three express homologous proteins, CD1a (49kd), CD1b (45kd) and CD1c (43 kd) molecules respectively.[86] These subsets are found on cortical thymocytes, dendritic cells and a subset of B cells. These antigens can be detected serologically and many different monoclonal antibodies are available for each subgroup.[87] Langerhans cells express CD1a molecules at exceptionally high levels with virtually no CD1b and only modest CD1c expression, whereas other dendritic cell subsets predominantly display CD1b molecules with varying degrees of CD1a and CD1c expression.[83]

A positive staining reaction with CD1a (also called OKT6/Leu6) is considered by some as the **gold standard** for Langerhans cell identification, despite the fact that this epitope is also present in the cortical thymocyte because, with maturation, cortical thymocytes lose CD1a before exiting the thymus.[45, 88]

3.2.6: Tissues in which Langerhans cells have been found:

Langerhans cells have been found in a wide variety of tissues, apart from the epidermis.[88] To site a few examples, the buccal mucosa,[89] oesophagus,[90] lungs,[10, 11, 88] female genital tract,[91, 92] conjunctiva,[93] palatine tonsil,[94] nose,[95, 96] larynx and hypopharynx,[97] vocal cords,[98] hair follicle,[99] etc.

Langerhans cells have also been found to secrete a variety of cytokines that are important in the pathogenesis of contact dermatitis, atopic dermatitis,[100, 101] histiocytosis X, HIV-1 infection and skin graft rejection.[88, 102]

Increased numbers of Langerhans cells have been found associated with a number of conditions[88] like lichen planus, [103-105] in the peripheral blood of patients with burns and trauma,[106, 107] in the epithelial surface of the lower respiratory tract in cigarette smokers,[108] in the conjunctiva of patients with contact dermatitis,[60, 109, 110] etc. Langerhans cells have also been implicated in the etiology of neoplastic transformation in immunocompromised individuals.[111]

The numbers of Langerhans cells have been found to be decreased in various conditions like cutaneous lupus erythematosus,[112] ultraviolet radiation,[113]

ageing[114, 115] skin carcinoma,[116, 117] basal cell carcinoma of face and trunk,[118] cervical condyloma and intra epithelial neoplasm,[119] etc.

3.3: CHRONIC OTITIS MEDIA:

The diagnosis of chronic otitis media implies a permanent abnormality of the pars tensa and pars flaccida, most likely a result of earlier otitis media, negative middle ear pressure or otitis media with effusion. Chronic otitis media equates with the earlier term chronic “suppurative” otitis media that is no longer advocated as chronic otitis media is not necessarily a result of gathering of pus.[120]

Classically, chronic otitis media is divided into 2 types:

Tubotympanic disease: also called safe or benign type. It usually has less severe complications.

Atticoantral disease: It is also called unsafe or dangerous type because of the complications associated with it.[121]

In the tubotympanic variety of otitis media, there is chronic inflammation within the mucosa of the middle ear and mastoid, with varying degrees of oedema, submucosal fibrosis, hypervascularity and infiltration with lymphocytes, plasma cells and histiocytes. Areas of the mucosa may ulcerate with proliferations of the blood vessels, fibroblasts and inflammatory cells, leading to the formation of granulation tissue. There is production of mucopurulent discharge which drains via a tympanic membrane perforation. The mucosal changes may progress and coalesce to form aural polyps that can protrude

through defects in the tympanic membrane. Thus, simple closure of a perforation in active mucosal chronic otitis media without surgical removal of infected mucosa and granulation tissue from the mastoid is fraught with failure to control the disease.[120]

In the atticoantral variety of chronic otitis media, also known as cholesteatoma, the hallmark is its retention of keratin debris. Thus, a “keratoma” would be, histologically, a more accurate term. The squamous epithelial lining or “matrix” of a cholesteatoma is similar to that of skin. The matrix is usually surrounded by a layer of inflamed, vascular, subepithelial connective tissue. A cholesteatoma can be filled with keratin and be quite dry, or be associated with active bacterial infection, leading to profuse, malodourous otorrhoea. Cholesteatomas are potentially dangerous because of their potential to incite resorption of bone, leading to intratemporal and intracranial complications.[120]

The light and electron microscopic findings in a human cholesteatoma matrix have been studied in detail. The cellular composition of the matrix and its adjacent mucosal lining is not static but undergoes permanent changes in terms of cells moving from the circulation in and out of this area. Migrant lymphoid and non lymphoid cells (Langerhans cells, monocytes) presumably play a key role in the natural history of the disease.[40]

Understanding of the destructive properties of cholesteatoma has been progressively clarified in the last few decades. Theories of avascular pressure necrosis have been replaced by the concept that complex cellular and chemical events of chronic inflammation induce bone breakdown. Mononuclear

inflammatory cells in the subepithelial granulation tissue zone of the cholesteatoma generate collagenase and lysosomal enzymes. Prostaglandins and other demineralizing factors also participate in the localized bone resorptive process. Epidermal Langerhans cells also play an important role. It appears that Langerhans cells initiate an immunologic response in the presence of antigens. The outcome of this reaction is an inflammatory reaction. In cholesteatoma, the inflammatory reaction is unable to correct the anatomical deformity of the retraction pocket. Therefore, it becomes chronic. This may stimulate a local hypersensitivity state and further propagation of the inflammation. New knowledge of the immunologic function of the epithelium, especially of epidermal Langerhans cells, may enable us to determine the role of the epithelium in cholesteatoma formation.[122]

3.4: CURRENT KNOWLEDGE BASED ON RESEARCH DONE ON THE TYMPANIC MEMBRANE:

3.4.1: Structure of the tympanic membrane:

The tympanic membrane separates the tympanic cavity from the external acoustic meatus. It is thin, semi-transparent and almost oval, somewhat broader above than below. It lies obliquely at an angle of about 55° with the meatal floor. At most of its circumference is a thickened fibrocartilaginous ring or annulus which is attached to the tympanic sulcus at the medial end of the meatus. The sulcus is deficient superiorly, resulting in the formation of the anterior and

posterior mallear folds from the edges of the notch to the handle of the malleus. The **pars flaccida** is the small triangular part of the membrane which lies above these folds, and is lax and thin. The rest and major part of the membrane is the **pars tensa** which is taut.[123]

There have been numerous investigations on the structure of the tympanic membrane under light and electron microscopy.

Histologically, it is composed of three strata: an outer cuticular layer, an intermediate fibrous layer and an inner mucous layer. The cuticular stratum is continuous with the thin skin of the external acoustic meatus. It is keratinized, stratified squamous in type and is hairless. The mucous stratum is part of the mucosa of the tympanic cavity, consisting of a single layer of flat cells.[123]

Lim DJ[124, 125] described the ultrastructure of the pars tensa and pars flaccida of the tympanic membrane in guinea pigs, cats, squirrel monkeys, rabbits, and sheep. Lildholdt et al[126] described the ultrastructure of the tympanic membrane in the rhesus monkey. Johnson et al[127] and Schmidt and Hellstrom[128] also described its ultrastructure in the guinea pig. Detailed descriptions of the human tympanic membrane were done by Hentzer[129] and Lim DJ.[130] A summary of their findings is as follows.

In the **pars tensa** of the tympanic membrane, three layers were distinctly recognized, an outer epidermal, a middle lamina propria and an inner mucous layer. The epidermis was reported to be divided into (1) stratum corneum, (2) stratum granulosum and (3) stratum Malpgii, which was further divided into stratum spinosum and stratum basale. There was no evidence of a stratum

lucidum. The epithelium was said to rest on a continuous basement membrane was reported to lack the characteristic epidermal rete pegs seen in the skin.

The lamina propria was subdivided into (1) subepidermal connective tissue, (2) radiate collagenous bundle layer, (3) circular collagenous bundle layer and (4) submucosal connective tissue layer.

The mucous layer was described to be composed of simple squamous to cuboidal epithelial cells, sometimes with and sometimes without cilia.

Pars Flaccida: In 1830, Shrapnell first described the pars flaccida of the mammalian tympanic membrane and hence it was named after him (**Shrapnell's membrane**).[131] He described this portion of the tympanic membrane as being more elastic than the pars tensa. Some subsequent authors felt that this part of the tympanic membrane lacked a lamina propria. However, Lim DJ[125] described the pars flaccida of the tympanic membrane as having three layers, like the pars tensa. He said the epidermal and mucosal layers were similar in structure to that of the pars tensa and that the lamina propria was present. However, the compact fibrillar bundles of radial and circular fibres were missing completely. There was no clear-cut morphological distinction between the subepidermal and submucosal connective tissue layers.

In a comprehensive electron microscopic study of human tympanic membrane, Lim DL et al,[130] in 1970, described in the basal portion of epidermis a cell that did not possess characteristics of an epithelial cell. In retrospect it is thought that this cell was probably a dendritic cell.

3.4.2: Studies involving Langerhans cells in the tympanic membrane:

Animal experimental studies:

Forséni et al[132] studied the tympanic membrane in rats. In this study, experimental otitis media was produced in Sprague Dawley rats by inoculation with Streptococcus pneumoniae, acute otitis media was confirmed by observing the tympanic membrane with an operating microscope. A temporal study was done on days 3, 6 and 10, using immunocytochemical markers to map out the inflammatory cells seen. The results of this study with regards to the dendritic cells was that their numbers were maximum on day 3, still abundant on day 6 and fewest on day 10, but these cells were noted maximally in the subepithelial connective tissue. Very few were seen in the epithelial layer. **The normal tympanic membrane was not seen to have these cells.**

Ichimiya et al[133] studied the immunological potential of the tympanic membrane in mice by staining normal and inflamed tympanic membrane by immunocytochemical techniques. Here healthy BALB/c mice were used and experimental otitis media induced on one side. Quantitative assay of IgA positive, CD4 positive, CD1a positive and mast cells was done in both infected and control ears. Observations were made in the middle ear mucosa as well as the tympanic membrane. In this study, the normal tympanic membrane showed far less infiltration than the middle ear mucosa. **CD1a positive cells were absent in the normal tympanic membrane except in the pars flaccida, and in the manubrial and annular parts of the pars tensa.** The rest of the pars tensa was devoid of Langerhans cells. In the tympanic membranes in which otitis media had

been induced, there was an overall increase in CD1a positive cells. The pars tensa this time showed cells. There were also cells seen in the subepithelial tissue. Overall, the middle ear mucosa showed far more cells than the tympanic membrane. In this study, quantitative analysis of cells was done, comparing Ia, mast, CD4 and IgA positive cells. They concluded that the pars flaccida, and the manubrial and annular parts of the pars tensa are immunologically potential sites. The rest of the tympanic membrane shows immunologic response because of migration of Langerhans cells through the tympanic membrane following antigenic stimulation.

Summary of studies on Langerhans cells in the human tympanic membrane and middle ear tissue:

The poor availability of fresh tympanic membrane tissue makes studies on the normal tympanic membrane difficult. Studies have been done, however, in specimens removed from diseased ears. Langerhans cells have been demonstrated in large numbers in the matrix of aural cholesteatomas.[40, 73, 134-137] They have also been found in inflamed tympanic membranes and middle ear mucosa.[138-140] Based on preceding animal experimental studies, the normal tympanic membrane was hitherto thought to be devoid or almost devoid of these cells. Two studies have been done on normal cadaveric tympanic membrane, one by Hussl et al,[37] and the other by Gantz.[122] Interestingly, these have contrasting findings. These, along with research done on Langerhans cells in diseased human tympanic membrane are described below.

Palva et al[59] studied inflammatory cell subpopulations in chronic otitis media. Tissues studied were cuboidal or secretory epithelium, ear canal skin and Shrapnell's membrane, cholesteatoma membrane and open cavity skin in revision ears. CD1a positivity and HLA DR positivity were tested for. This study showed Langerhans cells in the secretory epithelium, subepithelial lymphoid nodules and cholesteatoma epithelium, although they were not seen in the canal skin. One Shrapnell's membrane had no cells, while the other had.

Veldman[40, 133] studied the immunology of cholesteatoma and compared human cholesteatoma matrix tissue with normal tympanic membrane and external ear skin. Membrane ATPase staining of matrix and submucosal layers of cholesteatoma tissue showed the extensive presence of characteristic Langerhans cells with their dendritic processes in the epithelial layer. **The normal tympanic membrane contained hardly any detectable Langerhans cells**. The external ear skin showed a picture similar to that of normal skin. Veldman[40] stated that since these cells are hardly present or even absent in the normal tympanic membrane, it could be tentatively concluded that they follow a change in the nature of such an epithelial lining like an antigenic stimulus, retraction pocket or perforation with infection and subsequently enter. He also stated that the migratory properties of Langerhans cells would mean that they not only move into but also wander out of the matrix back into the patients' lymphoid system. Local production of lymphokines in the Langerhans cell-T cell microenvironment would possibly have particular consequences like triggering of bone resorption and destruction. If an "irritant" (antigen/hapten) together with the

local physical state of the tympanic membrane-middle ear complex were responsible for the development of the cholesteatoma, then sensitization of the lymphocytes of the T lineage would also occur, including memory cell production. He concluded that this knowledge was clinically very relevant as meticulous surgery to eradicate cholesteatoma, would therefore, not interfere with cells already in circulation.

Studies by van Dijk et al[138] showed that Langerhans cells probably play a key role in skin-related disorders, including cholesteatomas. He postulated that they originate from a mobile cell population of monocyte origin and migrate into and out of the body's lining. Since their custodial function is often carried out in close relation with T-lymphocytes, he used monoclonal antibodies against Langerhans' cells and T-lymphocyte membrane receptors, which **revealed the presence of these cell populations in cholesteatoma matrices but not in the tympanic membrane**. He thus said that the Langerhans cells and T-cell traffic through cholesteatomas have an important role in relation to the pathogenesis, natural course and recurrence of cholesteatomas.

Shinoda et al[141] tried localizing intercellular adhesion molecule-1 (ICAM-1) in middle ear cholesteatoma. ICAM-1 appeared to be localized on keratinocytes in all layers of the epithelium and on Langerhans cells in both the epithelium and granulation tissue of cholesteatoma. ICAM-1 was not found in the epidermis of normal external ear canal skin, normal tympanic membrane or normal facial skin. This suggested that ICAM-1 may have a role in clinical development of

cholesteatoma, including migration, adhesion and proliferation of lymphocytes, Langerhans cells and keratinocytes.

Park[58] studied the occurrence rate of Langerhans cells in cholesteatoma matrix, cavity skin and posterior auricular skin, to evaluate their pathological significance. He found that the cholesteatoma matrix was varying in thickness, specimens from subjects with the clinical symptom of otorrhoea, tending to have thicker epithelium with epithelial and subepithelial inflammatory cell infiltration. Numerous Langerhans cells were found in the cholesteatoma matrix, cavity skin and posterior auricular skin specimens. **Langerhans cells in the cholesteatoma and cavity skin were more numerous and had longer dendritic processes** than those in the preauricular skin. Also, there tended to be direct correlation between the number of Langerhans cells and the degree of the subepithelial inflammation in the cholesteatoma.

In the study done by Palva and Taskinen,[59] CD1a positive cells were found in the subepithelial connective tissue and in lymphoid follicles in the middle ear mucosa of cases with chronic otitis media, with and without cholesteatoma.

Takahashi and Nakano[73] demonstrated Langerhans cells in cholesteatoma using antiserum against S100 protein. Control specimens were normal skin from the external ear and from tympanic membranes of patients undergoing surgery for chronic otitis media. They found Langerhans cells containing S100 protein immunoreactivity scattered in the epithelium of the ear skin and tympanic membrane with otitis media but restricted to stratum spinosum. No cells were found in the subepithelial connective tissue. Also, they

had simpler shape and had fewer dendritic processes than those found in aural cholesteatomatous tissue. When the cholesteatomatous tissue was studied, numerous S100 positive dendritic cells were recognized. There was an obvious increase. However, in these cases only the cholesteatomatous tissue were studied and not the tympanic membranes. Those cases with ottorrhoea had more cells and the epithelium was thicker. All the cells in the cholesteatomatous tissue had **well developed dendritic processes, which branched out intricately**. **The processes were mainly directed towards the epithelium**. Cells were also found in the subepithelial connective tissue. In the cases with ottorrhoea, there were areas in the subepithelium with massive lymphocytic infiltration with S100 positive cells in these infiltrates. On quantification, all controls had less than 20 Langerhans cells per mm of epithelium, in cholesteatoma with otorrhoea, 33 per mm and in cholesteatoma without otorrhoea, 14 per mm. He described the role of the Langerhans cells as being antigen presenting cells at these sites. And the co-localization of Langerhans cells and lymphocytes in the subepithelial region of the cholesteatoma suggested that these cells play an important role in the immunodefence system.

In a study done by Hussl et al[37] on normal tympanic membranes, 12 normal cadaveric tympanic membranes from patients who had died of causes other than ear disease and had had no history of middle ear diseases, removed 14-21 hours after death, were studied. The epidermal layer was peeled off and stained with the following antibodies: HLA-DR, OKT6/CD1a and LAG. The results of this study showed that in the epidermis of all 12, a dense network of cells

could be demonstrated which were **positive for HLA-DR but negative with CD1a and LAG antibodies.** However the number of HLA-DR positive cells per high power field when compared with retroauricular skin was lower. **This was however not quantified.** On electron microscopy, the structure was found to be similar to Langerhans cells except that **no Birbeck granules were seen**. In tissue culture studies typical veiled cells could be recovered from the culture medium. They were comparable to defined types of mature dendritic cells such as proliferating dendritic cells from the peripheral blood.[142] In this study,[37] possible explanations given for CD1a negativity in the normal tympanic membrane were: 1. Dendritic cells in the tympanic membrane may have a different phenotype than epidermal Langerhans cells, 2. Antigens or epitopes like CD1a or LAG could possibly disintegrate postmortem (>14 hours after death).

Gantz[122] looked at epidermal Langerhans cells in cholesteatoma matrix and compared them with normal tympanic membrane and normal ear canal skin. Normal ear skin demonstrated Langerhans cells in the suprabasal portion of the epithelium. Cell bodies were round with variable number of filamentous dendritic processes. Electron microscopy showed Birbeck's granules. The stratified squamous epithelium of the normal tympanic membrane also contained Langerhans cells, but they were fewer in number than in the ear canal skin and had an elongated appearance. Although Gantz reported the presence of these cells in the normal tympanic membrane, **quantification of the cells per unit length or quantitative comparison between inflamed tympanic membrane was not done**. The other different finding was that Langerhans cells were evenly

distributed through the entire surface of the tympanic membrane and not limited to the pars flaccida and the annular and manubrial regions of the pars tensa, as has been suggested by others like Ichimiya et al.[133, 143] **Cholesteatoma matrix showed increased number of Langerhans cells** compared to the other two (again quantification not done). In the cholesteatomatous tissue, he reported that the Langerhans cells were at variable levels of the epithelium and not confined to the suprabasal portions. On electron microscopy, the cells looked strikingly different from the normal tissue. They were elongated cells, closely apposed to lymphocytes. Increased mitochondria, multiple golgi apparatus and increased secretory vacuoles indicated increased secretory activity.

Veldman et all,[138] Gantz[122] and Takahashi and Nakano,[73] all suggested that Langerhans cells within the cholesteatoma matrix were responsible for generating and maintaining the chronic immunological reactions of this disease while Kaehoene et al[139] and Palva and Taskinen[59] did not support the hypothesis of Langerhans cells having a primary role in the development of cholesteatoma.[58]

Gantz[122] hypothesized that the keratin, intracellular debris, and bacteria in the retraction pocket of cholesteatomas might be recognized as nonspecific antigens by epithelial Langerhans cells, which bound and presented them to lymphocytes either in the epithelium or regional lymphnodes, generating a cytotoxic T cell response directed at the initial site of sensitization. Even though the specific mechanism of activation is unknown, it appears than Langerhans cells in cholesteatoma matrix could be an important factor in activating and

maintaining a chronic inflammatory state in cholesteatomas, resulting in connective tissue breakdown and bone resorption. Veldman et all[138] found an abundant Langerhans cells population not only in the cholesteatoma matrix, but also in the subepithelial tissue. In his opinion, this could be the route for the mobilized Langerhans cells on their way to the squamous epithelium. Palva and Taskinen[59] did not agree with Veldman's systemic concept of cholesteatoma. Their observation of Langerhans cells in the secretory epithelioum of subjects with secretory otitis media and chronic otitis media, in which there was not the slightest sign of keratinisation argued against the specific role for Langerhans cells in recurrent cholesteatoma. They felt that Langerhans cells should simply be viewed as normal defense cells. In the study done by Park[58] there was a marked difference in the number and shape of Langerhans cells according to the inflammatory condition of the subepithelial tissue of the cholesteama. He found that the more the inflammation, the more the number of Langerhans cells. Also the Langerhans cells in cholesteatoma matrix with more inflammation had also more numerous and longer dendritic processes than those in the normal post auricular skin or cholesteatoma matrix with less inflammation. This suggested an activated condition of Langerhans cells in the inflamed cholesteatoma matrix. Thus, he agreed with the view put forward by Palva and Taskinen[59] that Langerhans cells may be regarded as normal immune defense cells. However, their significant presence at inflammatory sites, suggested that these cells may be involved in the proliferation of existing cholesteatoma.

MATERIALS AND METHODS

4. MATERIALS AND METHODS

4.1. COLLECTION OF SPECIMENS:

Normal tympanic membranes were collected from cadavers donated to the Department of Anatomy, Christian Medical College, Vellore. The cadavers chosen for the study died of causes other than ear disease and had no known history of chronic ear disease. Collection of specimens was done within 48 hours from death.

Specimens of tympanic membrane were collected from patients undergoing tympanoplasty for chronic otitis media of the tubotympanic and atticoantral varieties, in the Department of ENT, Christian Medical College, Vellore. After acquiring patient consent, the freshened edges of the tympanic membrane, removed as a routine part of the surgery and deemed for discard, were collected.

One tympanic membrane collected was from an individual who had a traumatic perforation, who underwent a tympanoplasty for the same.

4.2. CALCULATION OF SAMPLE SIZE:

A morphometric analysis of Langerhans cells in the tympanic membrane with regard to number of cells per unit length of basement membrane has not been reported prior to this. Hence, a pilot study was carried out comparing one normal tympanic membrane with one tympanic membrane with atticoantral disease and five tympanic membranes with tubotympanic disease.

Based on the pilot data, the mean (standard deviation) of number of cells per 100μm length of basement membrane was 9.3(3.3) and 3.0(0.89) in the tubotympanic disease and normal groups respectively. Using 3.3 as the standard deviation for the two groups (normal group had only one sample) with α and β errors at 5% and 20% respectively, the sample size needed to be studied was a minimum of 6 in each group.

However, it must also be borne in mind that availability of normal tympanic membrane for study is extremely low, as only cadavers donated to the Anatomy department within 48 hours of death could be used. Since the number of controls is rare, the case- control ratio was divided as 9:1. That means a minimum of 16 cases and 2 controls were required to attain a total of 18 cases. However, this is the minimum number required to be studied.

The total number of samples collected for study was 60. Of these, only 33 had adequate epithelium for study. The numbers obtained in each group were:

Normals: 2

Tubotympanic disease: 27

Atticoantral disease: 3

Traumatic perforation: 1

4.3. FIXATION AND PROCESSING OF TISSUES:

The collected tissues were fixed in neutral formalin for 1 week. Processing of the specimens into paraffin blocks was done as per the standard technique of

dehydration in ascending grades of isopropyl alcohol, clearing in toluene and finally, impregnation and embedding in molten paraffin wax.

4.4. STAINING OF SECTIONS:

4 micron serial sections were floated onto pre-cleaned slides which were coated with poly L-lysine. These were then incubated in a 37° C oven for 24 hrs and then a 58° C oven overnight, before they were ready for staining.

4.4.1. Staining with haematoxylin and eosin:

In each biopsy, sample sections at regular intervals of the serial ribbon were selected for staining with haematoxylin and eosin stain as per the standard technique.

4.4.2. Immunocytochemal staining (CD1a):

In each biopsy serial sections in which the epithelium was clearly visible by haematoxylin and eosin staining, were selected for immunohistochemical staining. The technique used for immunohistochemical staining for CD1a was the Polymer-HRP detection system, which is a modification of the standard avidin-biotin peroxidase technique. However, this is a biotin free detection system.

The primary antibody used was pre-diluted purified mouse monoclonal antibody diluted in phosphate buffered saline, pH 7.6, containing 1% BSA and 0.09% sodium azide, of the Biogenex Company.

Also obtained from the Biogenex Company was a super sensitive polymer-HRP detection kit containing:

a. Peroxide block: 3% hydrogen peroxide in water
b. Power block reagent: Casein and propriety additives in PBS with 0.09% sodium azide, used as a protein blocking agent.
c. Super enhancer reagent: A catalyst reagent used to enhance the signal after primary antibody incubation.
d. Poly HRP reagent: Anti-mouse and anti-rabbit IgG, labeled with enzyme polymer in phosphate buffered saline.
e. Liquid DAB chromogen: Diamino benzidine wich is a sensitive HRP colorimetric chromogen.
f. Stable DAB substrate buffer: Tris buffer containing the peroxides and stabilizers.
g. Rabbit negative control and mouse negative control (non immune serum in phosphate buffered saline).

Principles of the polymer technique of CD1a staining:

The demonstration of antigens in tissues and cells by immunohistochemical staining is a two-step process involving first, the binding of an antibody to the antigen of interest, and second, the detection and visualization of the bound antibody by one of a variety of enzyme chromogen systems. The advantage of the polymer kit technique over the standard avidin-biotin system is that problems associated with endogenous biotin are completely eliminated.

Preparation of the buffers used:

EDTA buffer: This was used as the buffer during antigen retrieval.

For 2 litres of buffer,

- Tris: 12.1 grams
- EDTA: 1.493 grams
- Distilled water: To make up to 2 litres

The pH of the solution was adjusted to 9.0.

Tris Buffered Saline: This was used as the wash buffer during the staining procedure.

For 2 litres of buffer,

- Sodium chloride: 16 grams
- Tris: 1.210 grams
- 1 Normal Hydrochloric acid: 8 ml

The pH of the solution was adjusted to 7.6.

Steps of the staining procedure:

1. The PLL coated slides with the sections of tympanic membrane were dewaxed in xylene for 30 minutes.
2. They were then placed in 2 changes of 1 minute each of absolute isopropyl alcohol, to remove the xylene.
3. Slides were washed in running tap water for 10 minutes.

4. ANTIGEN RETRIEVAL: The process of fixation of tissue and processing into paraffin blocks masks the presence of antigenic epitopes, because of the formalin cross-linking that occurs. Thus, prior to immuno-staining, paraffin sections require to undergo a process known as antigen retrieval, whereby these formalin cross-links are broken and the antigens are free to react. Different primary antibodies have different techniques of antigen retrieval. The technique of antigen retrieval for the CD1a antigen employed involved pressure cooking as per the following steps.
    a. Sections were transferred to distilled water for 1-2 minutes.
    b. EDTA buffer was preheated by steaming in a pressure cooker without weight until steam escaped.
    c. The slides were arranged in a slide rack with adequate gap and then transferred to preheated buffer.
    d. This was then pressure cooked for 10 minutes at 120° C, 15 lbs pressure.
    e. The cooker was cooled to room temperature by plunging into a sink with water.
    f. The slides were then transferred to distilled water for 5 minutes.
    g. They were then transferred to Tris Buffered Saline (TBS) for 2 changes of 5 minutes each.

The remaining steps of staining were carried out in an air-conditioned room.

Fig. 1: Slides arranged in EDTA buffer for Antigen retrieval by pressure cooking

Fig. 2: Work table with some of the apparatus used in immunohistochemical staining

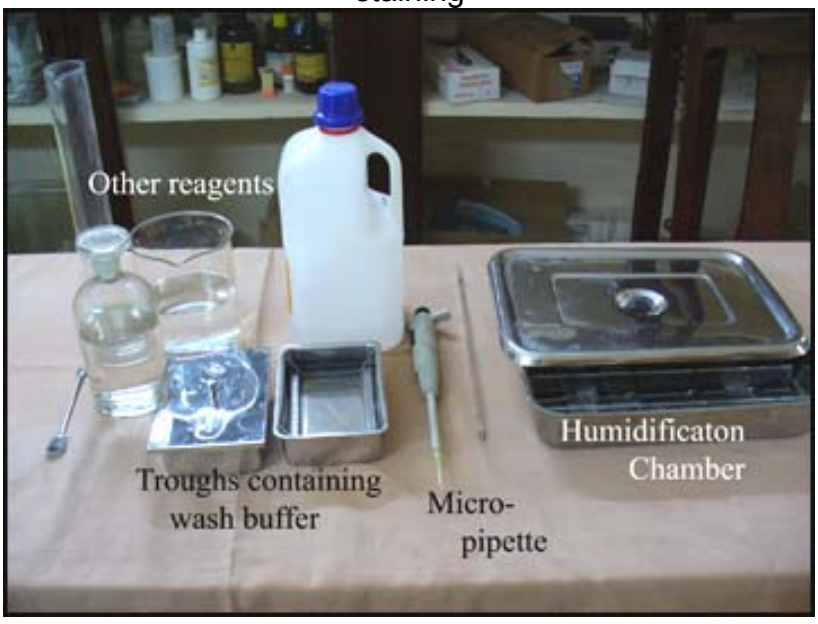
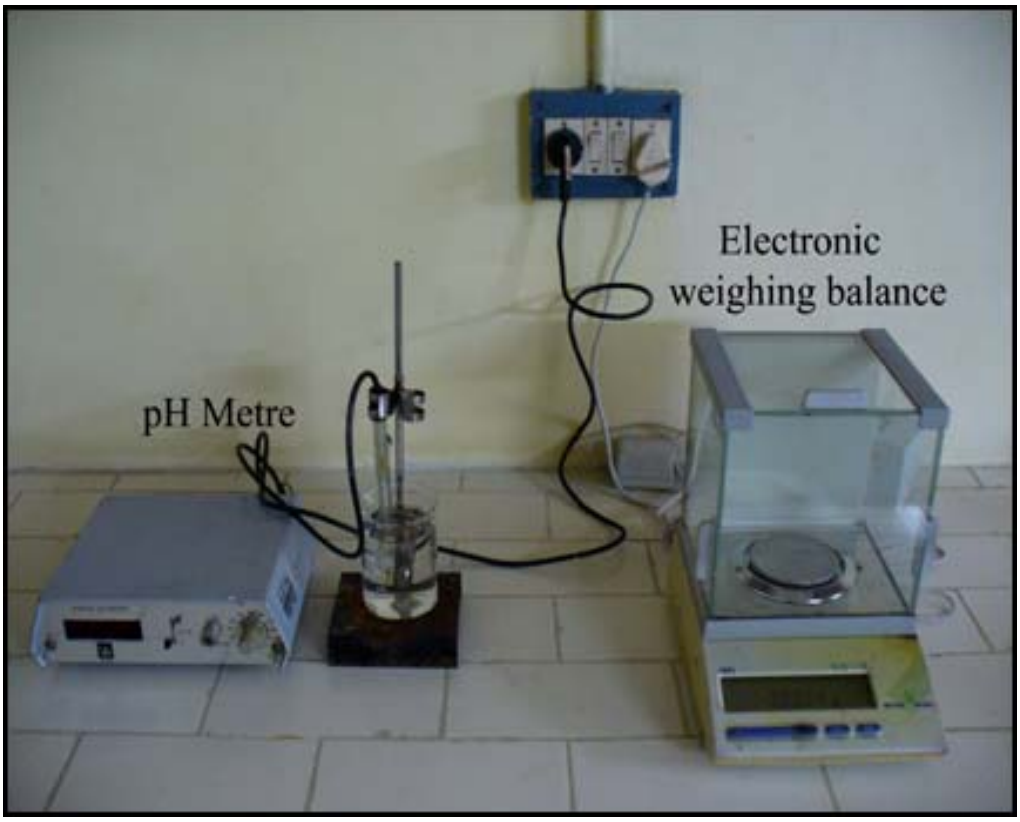


Fig. 3: Some equipment used during preparation of the buffers



5. PEROXIDASE BLOCK: This was used to block endogenous peroxidases. This step was carried out in a dark room to prevent decomposition of the hydrogen peroxide. Sections were covered with the peroxidase block solution and slides were placed in a humidification chamber for 10- 15 minutes.
6. The light was turned back on at this stage. Excess solution was drained away and transferred to Tris Buffered Saline for 2 changes of 5 minutes each.
7. POWER BLOCK: This was to block endogenous proteins. Sections were covered with the required amount of power block solution and slides placed in humidification chamber for 10 minutes.
8. Excess power block solution was wiped away but there was no TBS wash at this step.
9. PRIMARY ANTIBODY: The sections were covered with required amount of pre-diluted primary antibody (CD1a). They were arranged in the humidification chamber and left to stand for 1 hour.
10. Excess antibody was drained away and washed in Tris Buffered Saline 2 changes, 5 minutes each.
11. SUPER ENHANCER: This solution acted as a catalyst in the reaction. Sections covered with required amount of super enhancer solution and placed in humidification chamber for 30 minutes.
12. Excess solution drained away and washed in Tris Buffered Saline 2 changes, 5 minutes each.

13. POLY HRP REAGENT: This is the enzyme labeled antibody solution. Sections were covered with required amount of solution and incubated in the humidification chamber for 30 minutes.
14. Excess solution drained was away and the slides were washed in Tris Buffered Saline 2 changes, for 5 minutes each.
15. DIAMINO BENZIDINE: This step was the chromogen reaction to visualize the enzyme labeled antigen-antibody complex. Again, it was done in the dark to prevent degradation of hydrogen peroxide. 1ml of stable DAB buffer was mixed with 1 drop of DAB solution and sections covered with required amount of this solution. Slides were placed in humidification chamber for 5- 8 minutes.
16. Excess solution was drained away and washed in Tris Buffered Saline for 5 minutes.
17. Slides were washed in running tap water for 10 minutes.
18. COUNTER STAINING: This was done to achieve a nuclear stain for the cells in the sections.
    a. 2 dips in Harris Haematoxyline.
    b. Running water for 5 minutes.
    c. 2 dips in Lithium Carbonate for blueing.
    d. Running Tap water for 5 minutes to enhance blueing further.
19. Slides were then blotted, air-dried, dipped in xylem and mounted using DPX.

CONTROLS: For each batch of staining done sections of lymph nodes with Langerhans cell histiocytosis were used as positive controls. Negative controls were not used due to expense of the reagents.

4.5. LIGHT MICROSCOPIC STUDIES:

4.5.1: Slides stained with Eosin and Haematoxyline:

The sample slides which were stained with eosin and haematoxylin were used as guides to identify areas in the biopsy where the stratified squamous epithelium of the outer layer of the tympanic membrane was uninterrupted. In the normal tympanic membrane, the slides stained were studied to ensure that there were no features of acute or chronic inflammation.

4.5.2: Morphometric analysis of Langerhans cells done in the slides stained by immunocytochemistry (CD1a):

In the cases where the tympanic membrane was obtained during tympanoplasty surgeries, only the freshened edges from the margins of the tympanic membrane perforations were obtained. Thus the total length of tympanic membrane obtained in each case was only a few millimeters. Therefore, the decision was taken to calculate the number of cells present per 100μm length of basement membrane.

Slides were examined under a Leitz DMRHC Research microscope using semi-automated image analyzing software, Leica Qwin.

Fig.4: Leitz microscope connected to a computer with Leica Qwin semi-automated image analysis software used for measurements in this study

For each sample, 25 fields measuring 100μm were studied under high power (40X) magnification.

The parameters measured were:

1. The number of CD1a positive cells in which the nucleus was clearly visible for 100μm length of basement membrane.
2. In each specimen the horizontal diameters, measured parallel to the basement membrane of the stratified squamous epithelium, and the vertical diameters, measured perpendicular to it, of 10 cells were measured. Also measured in these cells were the number of dendritic processes and the length of each dendritic process.

4.6. ANALYSIS:

4.6.1: Qualitative Analysis:

The slides were studied to appreciate the morphology and pattern of distribution in the epithelium and subepithelial connective tissue and the interesting findings were described.

4.6.2: Statistical Analysis:

The mean number of cells per 100μm length of basement membrane was calculated in each of the 4 groups and these were compared using Kruskal Wallis test.

The mean diameter of each cell was calculated as the average of the horizontal and vertical diameters of the cell, horizontal diameter measured parallel to the basement membrane of the epithelium and vertical diameter measured perpendicular to it. Also studied, were the mean length of the dendritic processes and the mean number of processes per cell in each group.

The mean number of cells per 100μm length of basement membrane, the mean diameter of each cell, the mean length of dendritic processes and the mean number of dendritic processes per cell were compared between 2 groups by the non-parametric, Mann-Whitney U test.

RESULTS

5. RESULTS

5.1. QUALITATIVE ANALYSIS:

5.1.1. Sections stained with haematoxylin and eosin:

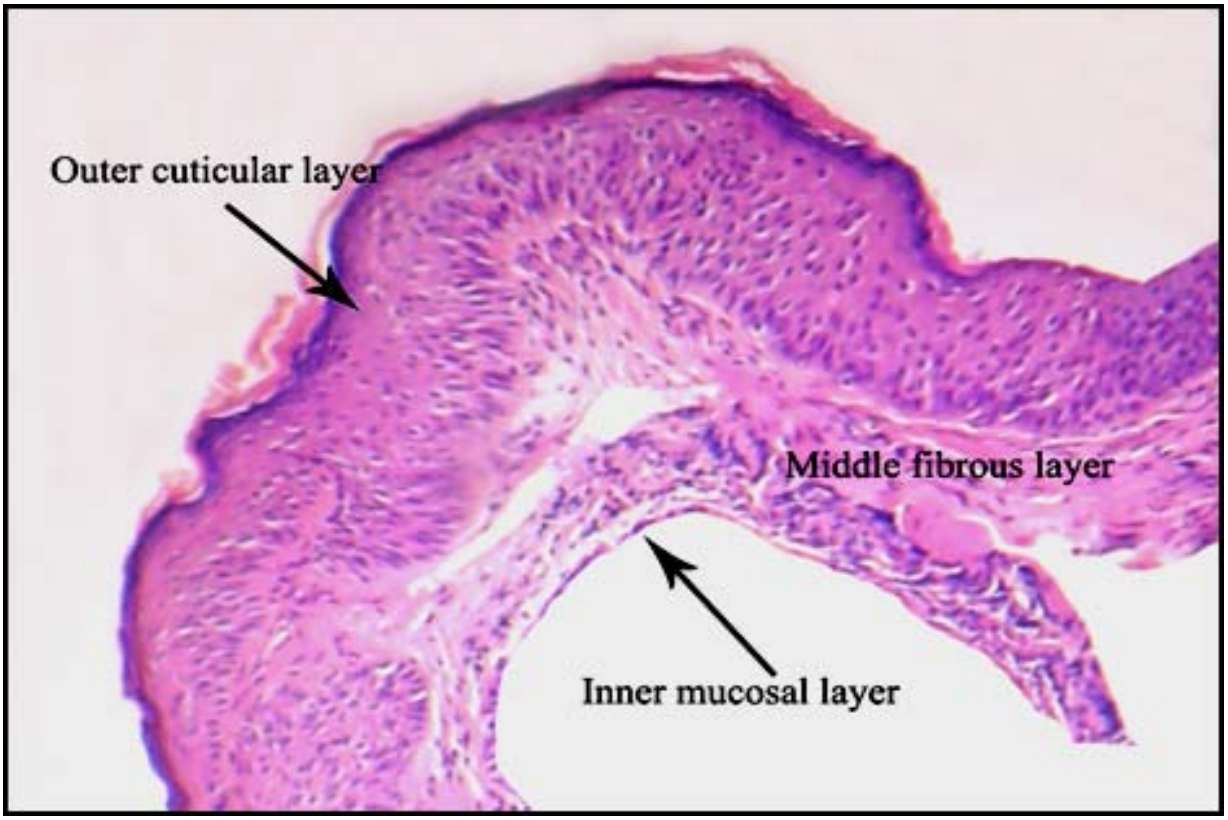
Out of the 60 samples that were processed for light microscopy it was found after staining with haematoxylin and eosin, that only 33 of these had adequate epithelium to be studied.

The normal cadaveric biopsies were examined and it was confirmed that there were no signs of acute or chronic inflammation.

In the tympanic membranes from cadavers with chronic otitis media of the tubotympanic variety, the epithelium was markedly thickened, with hyperkeratosis and rete pegs. There was moderately dense diffuse chronic inflammation which was lympho-plasmacytic in nature. There were variable degrees of fibrosis in the biopsies. Fig.5 shows a tympanic membrane of a patient with tubotympanic disease showing the layers of the tympanic membrane.

Similar inflammatory cells were seen in the biopsies with chronic otitis media of the atticoantral variety. However the epithelium was markedly hyper-keratotic and the granular layer was very prominent. Also, in these biopsies, the inflammation appeared focal and perivascular nature.

Fig.5: A biopsy of tympanic membrane from a patient with tubotympanic disease, stained with haematoxylin and eosin, viewed under low power. Note the hyperkeratosis, markedly thickened cuticular layer, chronic inflammatory cells and fibrosis



The biopsy of the tympanic membrane with the traumatic perforation looked remarkably like the normal tympanic membrane biopsies, without signs of lymphocytic infiltration.

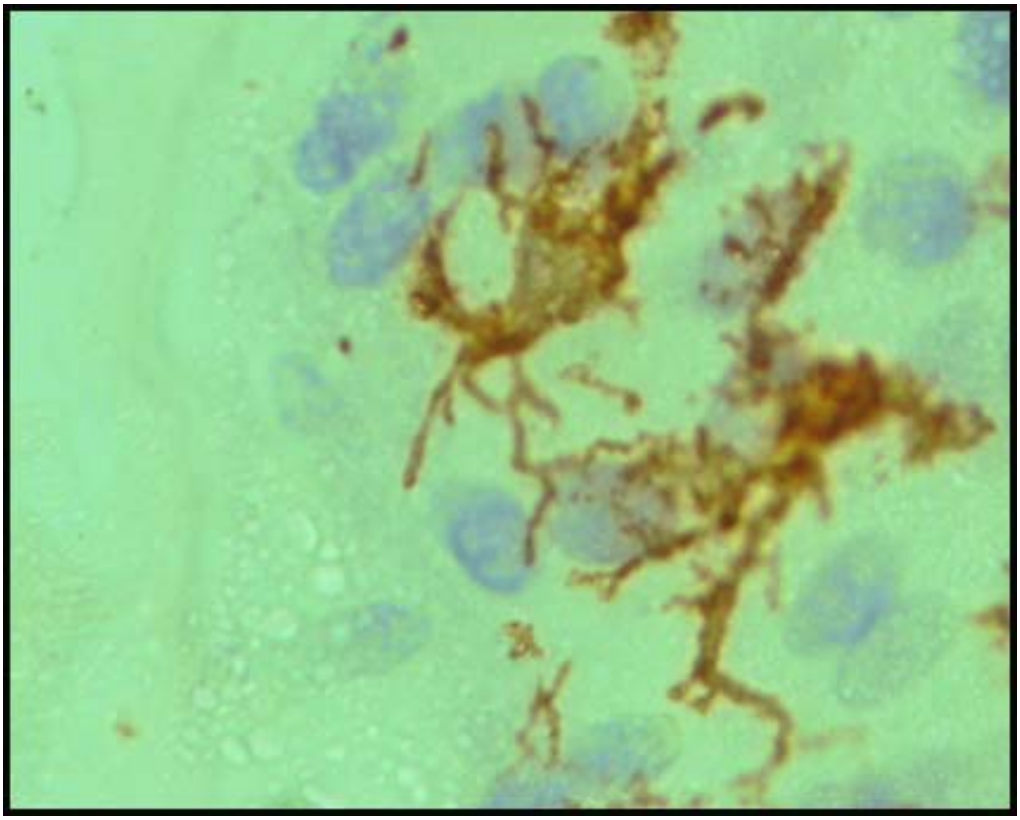
5.1.2. Sections stained by immunohistochemical technique:

All the 33 biopsies studied contained CD1a positive cells. The four categories looked at were normal tympanic membrane, tympanic membrane in tubotympanic disease, tympanic membrane in atticoantral disease and tympanic membrane after traumatic perforation.

The structure of the Langerhans cells seen was similar to those described in previous studies involving these cells. The cell body was round to ovoid with a large circular nucleus. From the cell body arose 1 to 6 dendritic processes. Several of these dendritic processes branched further, forming a highly interconnected network of processes. Many of these terminal processes ended in contact with the processes of other dendritic cells, showing the vast intercommunication between these cells (Fig. 6).

In the tympanic membranes studied, a variety of Langerhans cells were seen. In some areas, the dendritic processes were seen to be directed towards the surface of the stratified squamous epithelium in a manner similar to that described by Takahashi and Nakano[73]. However, this was not a uniform feature in the biopsies with cholesteatoma, as some processes appeared to be directed

Fig. 6: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin, viewed under oil immersion.
Note the branching dendritic processes which intercommunicate with each other.



towards other Langerhans cells and lymphocytes as well. Moreover, this feature was also seen in the biopsies with tubotympanic disease (Fig. 7 and 8).

Langerhans cells seen in the tympanic membrane were found to fall into similar groups to those described by Figueroa and Caorsi,[62] who conducted an ultrastructural and morphometric study of the Langerhans cells in the normal human exocervix. According to their classification, Langerhans cells could be dividied into 5 groups based on the number of dendritic processes they contained and the pattern of branching of these dendritic processes, as described below.

- Type I cells had a single, unbranched process (Fig. 9).
- Type II cells had a single process which in turn was branched (Fig. 9, 10 and 13).
- Type III cells were cells with 2 processes, irrespective of their branching pattern (Fig. 11, 12 and 13).
- Type IV cells had 3 or more dendritic processes (Fig. 14 and 15).
- Type V cells also had 3 or more processes, but these in turn branched intricately. (Fig. 16 and 17).

Another feature noticed was that although the CD1a positive Langerhans cells were mainly concentrated in the stratified squamous epithelial layer of the tympanic membrane, migration of these cells was noticed into the subepithelial connective tissue. Also, in the subepithelium, these cells were seen in close relation with aggregations of lymphocytes (Fig. 18, 19 and 20).

Figures 7 and 8: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin. Note the processes directed towards the surface of the stratified squamous epithelium.

Fig. 7: viewed under oil immersion.

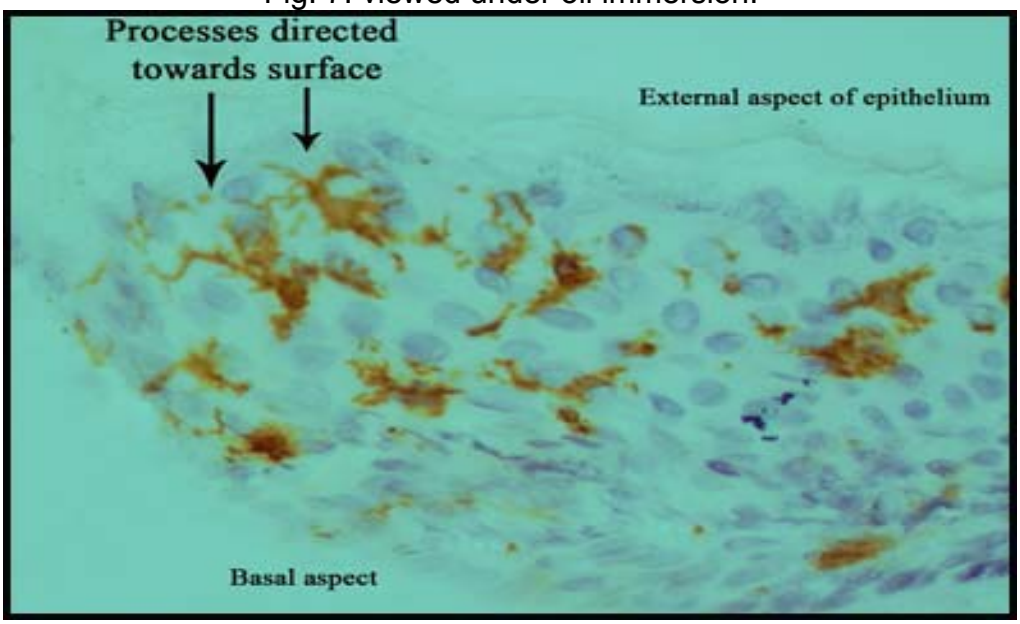


Fig. 8: viewed under oil immersion.

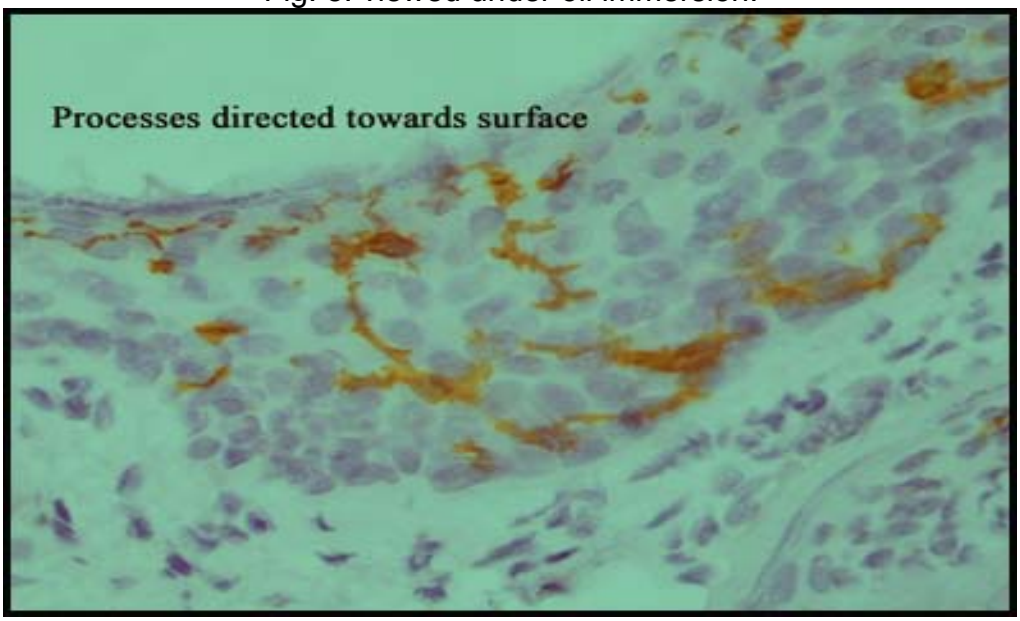


Fig. 9: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin. Magnification: 833
A type I cell with a single long unbranched process is seen along with a type II cell with a single process which divides into branches.

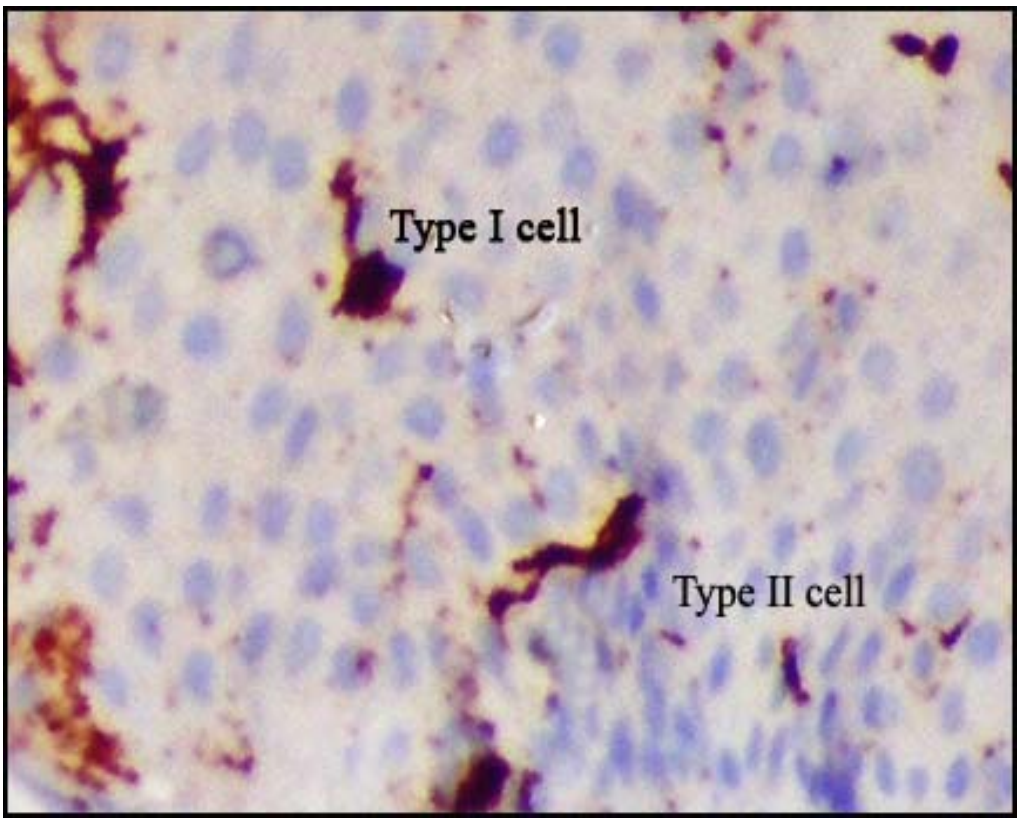
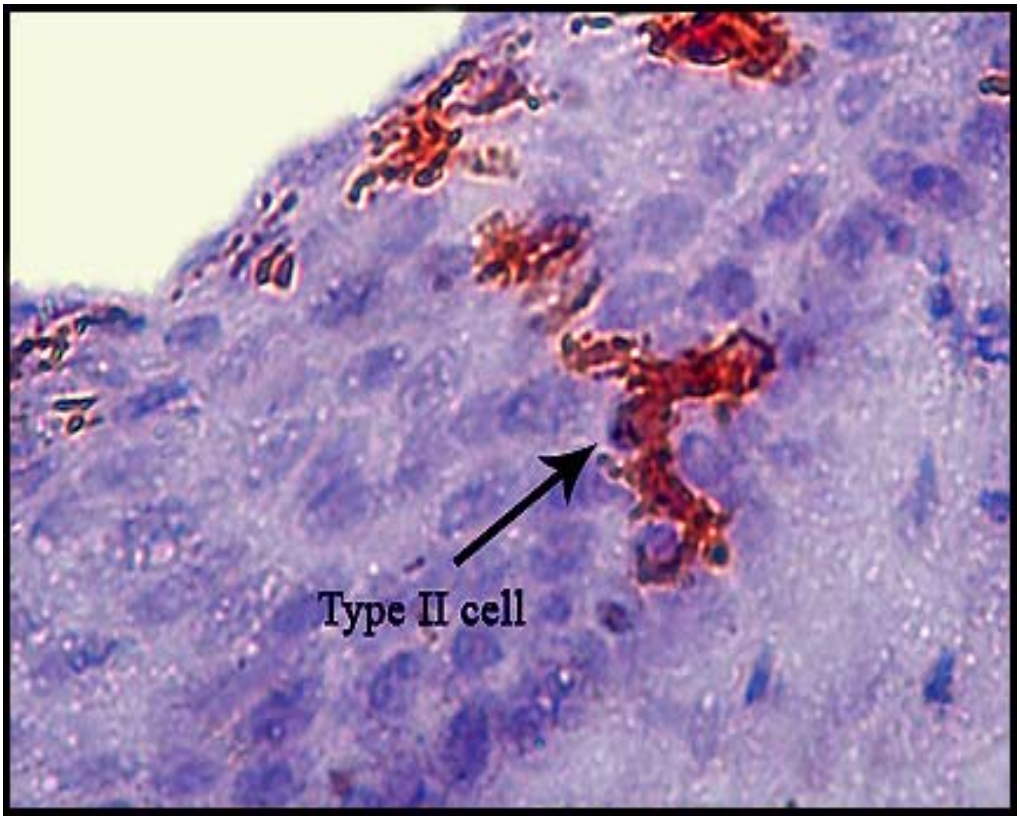


Fig. 10: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin. More type II cells with single, branching processes are seen

Viewed under oil immersion



Figures 11 and 12: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin. Type III cells, each having two dendritic processes are seen.

Fig. 11: Magnification: 667

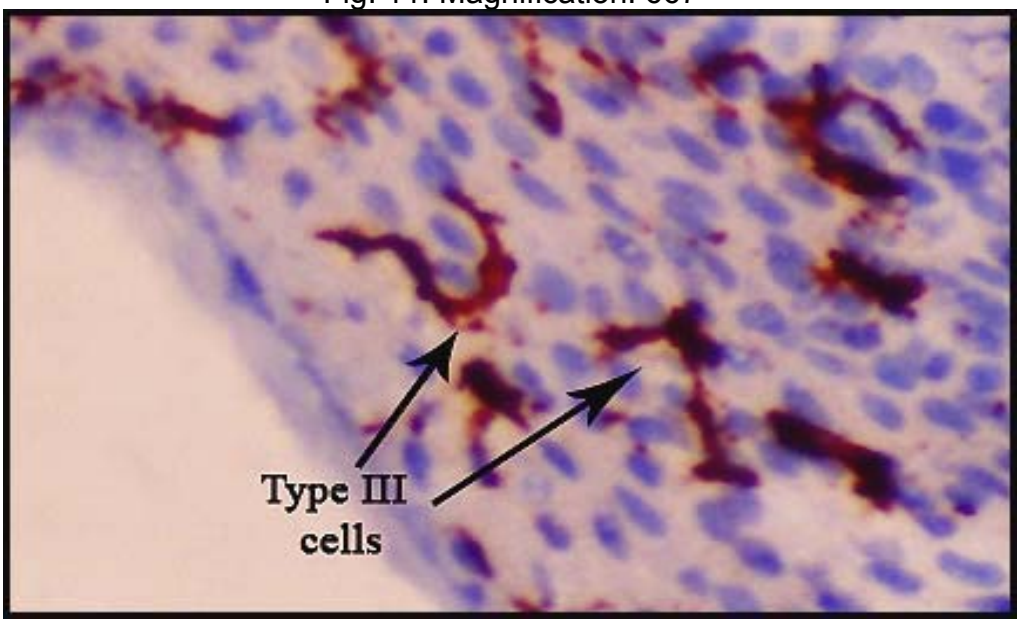


Fig. 12: Viewed under oil immersion

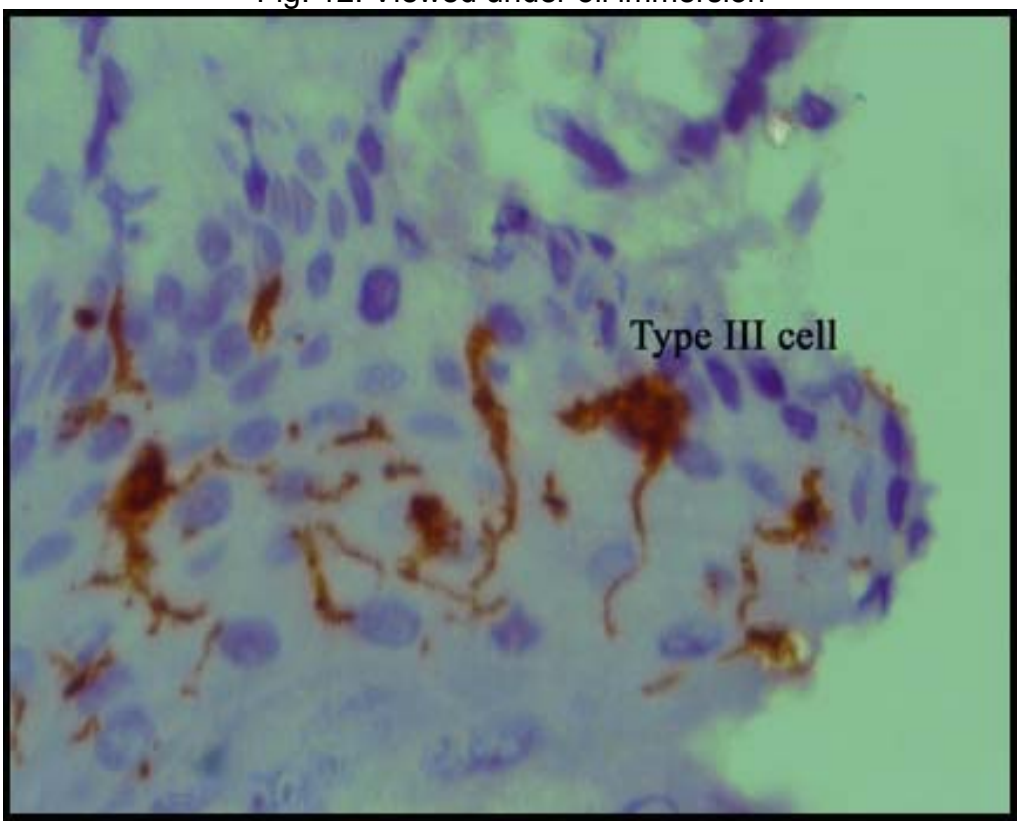
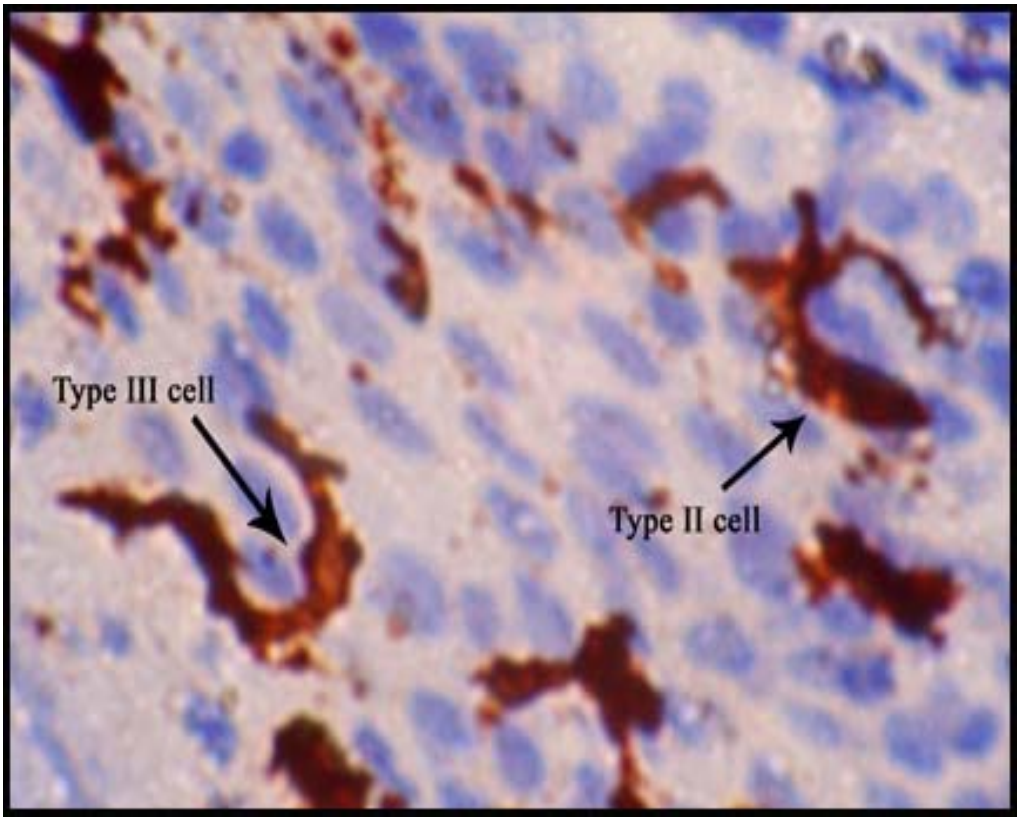


Fig. 13: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin, viewed under oil immersion. Type II and type III cells are seen.

Magnification: 1100



Figures 14 and 15: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin, viewed under high power
Type IV cells with 3 or more processes seen.

Fig. 14: Magnification: 367

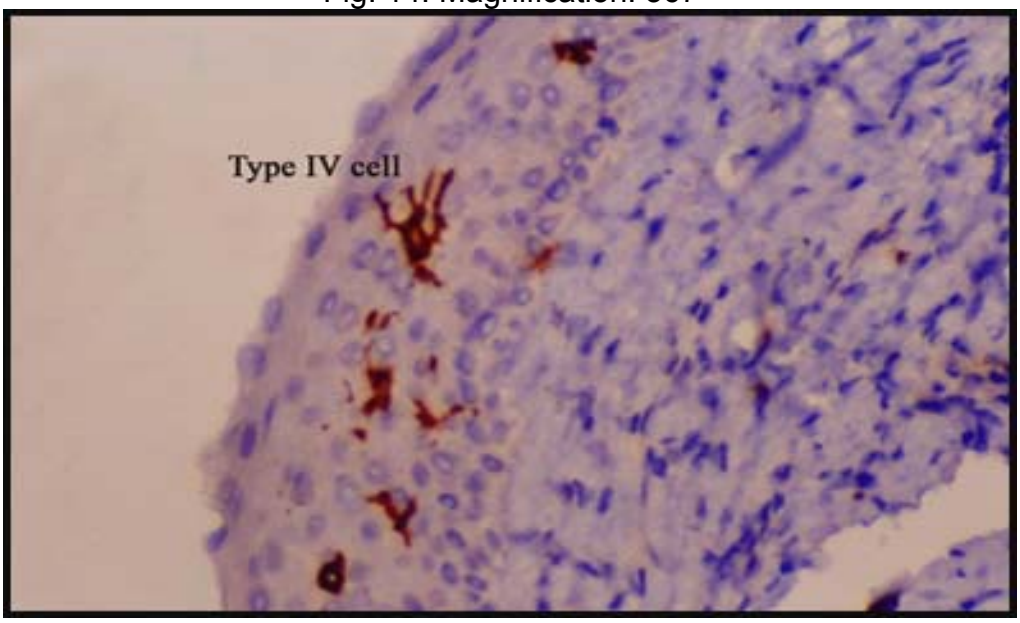
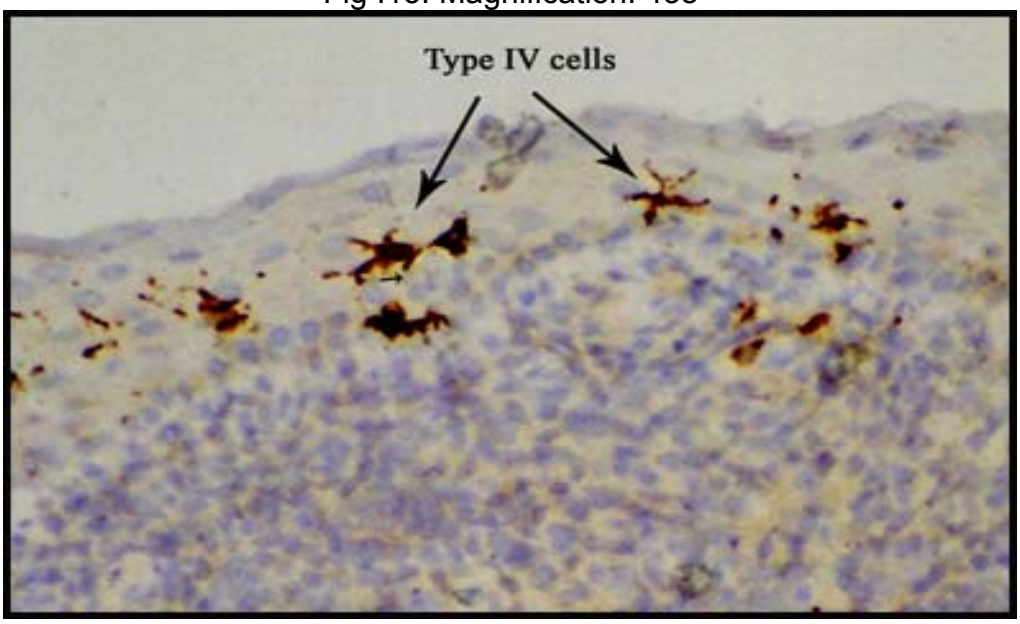


Fig .15: Magnification: 438



Figures 16 and 17: Tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin. Type V cells with 3 or more processes which further arborise seen.

Fig. 16: Viewed under oil immersion

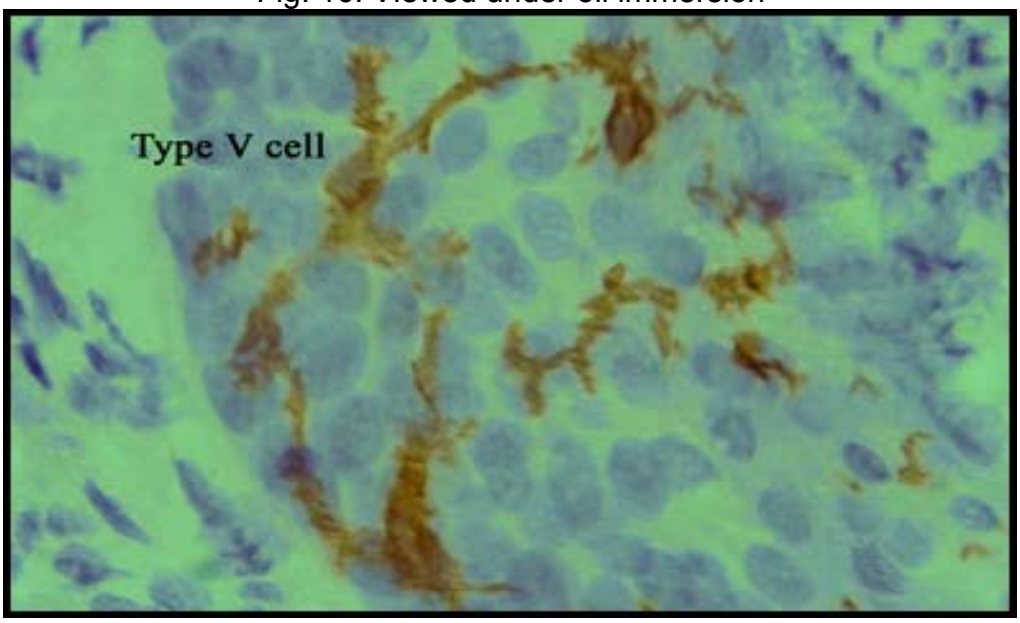
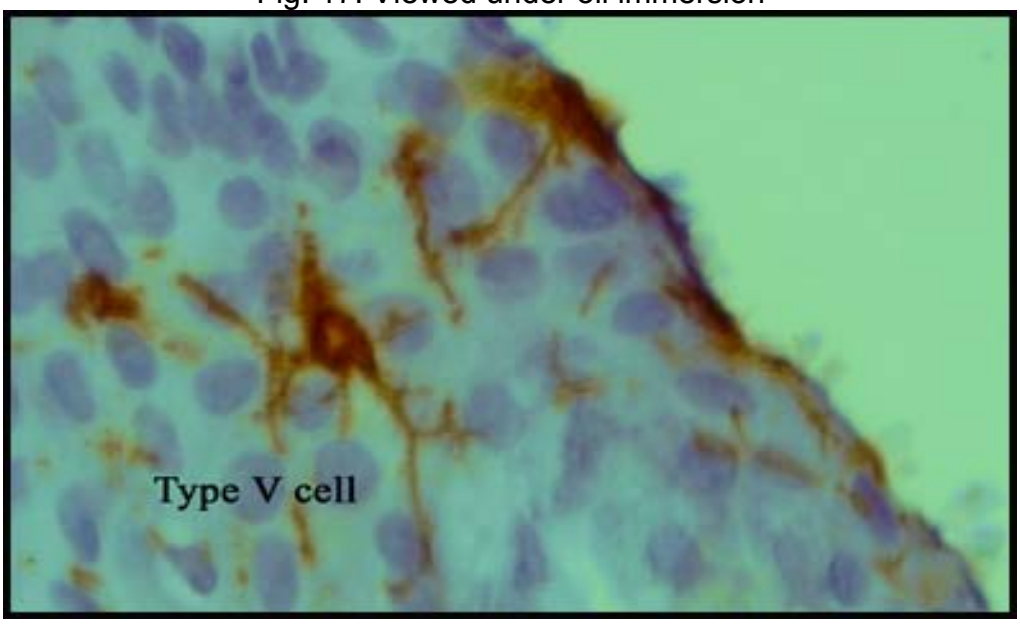


Fig. 17: Viewed under oil immersion



anic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin. Note the serial sections showing migration of the cells into the subepithelium and association with lymphoid aggregates

Fig. 18: Magnification: 133

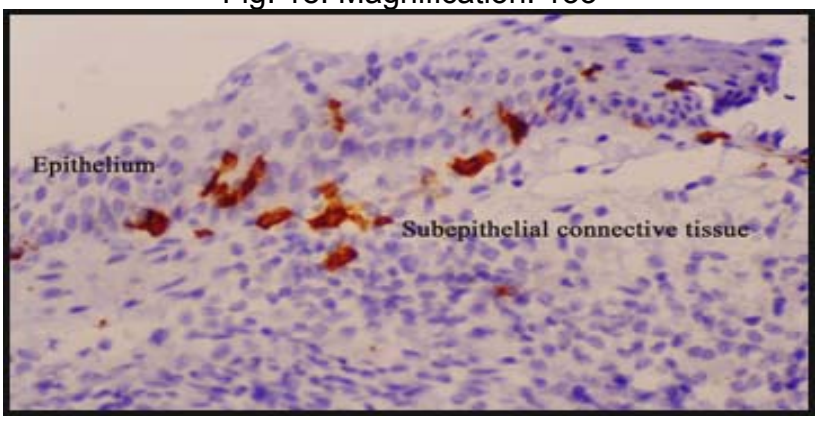


Fig. 19: Magnification: 225

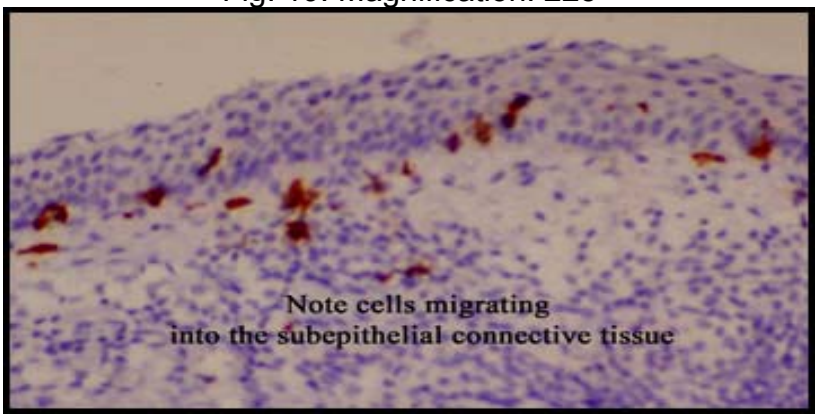
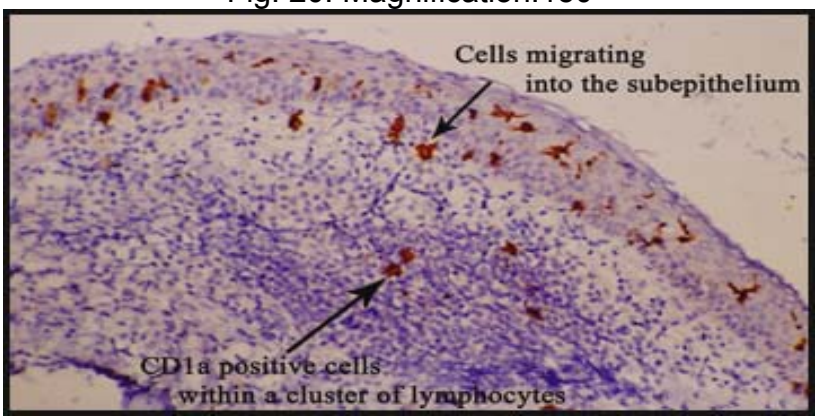


Fig. 20: Magnification:150



Interestingly, contrary to studies described earlier, the biopsies of the normal tympanic membranes studied contained CD1a positive Langerhans cells in their entire length. The cells appeared to be smaller and fewer in number than those with chronic otitis media.

Of the biopsies with chronic otitis media, those with atticoantral disease appeared to have more Langerhans cells than those with tubotympanic disease. These numbers were quantified and the data is presented in the tables that follow in the section on the statistical analysis. The following series of pictures shows the distribution of CD1a positive Langerhans cells in the tympanic membrane in the above conditions. (Fig. 21, 22 and 23)

Fig. 21: Normal tympanic membrane stained with CD1a antibody, counter-stained with Harris Haematoxylin, viewed under low power. Note the sparse but uniform distribution of Langerhans cells.

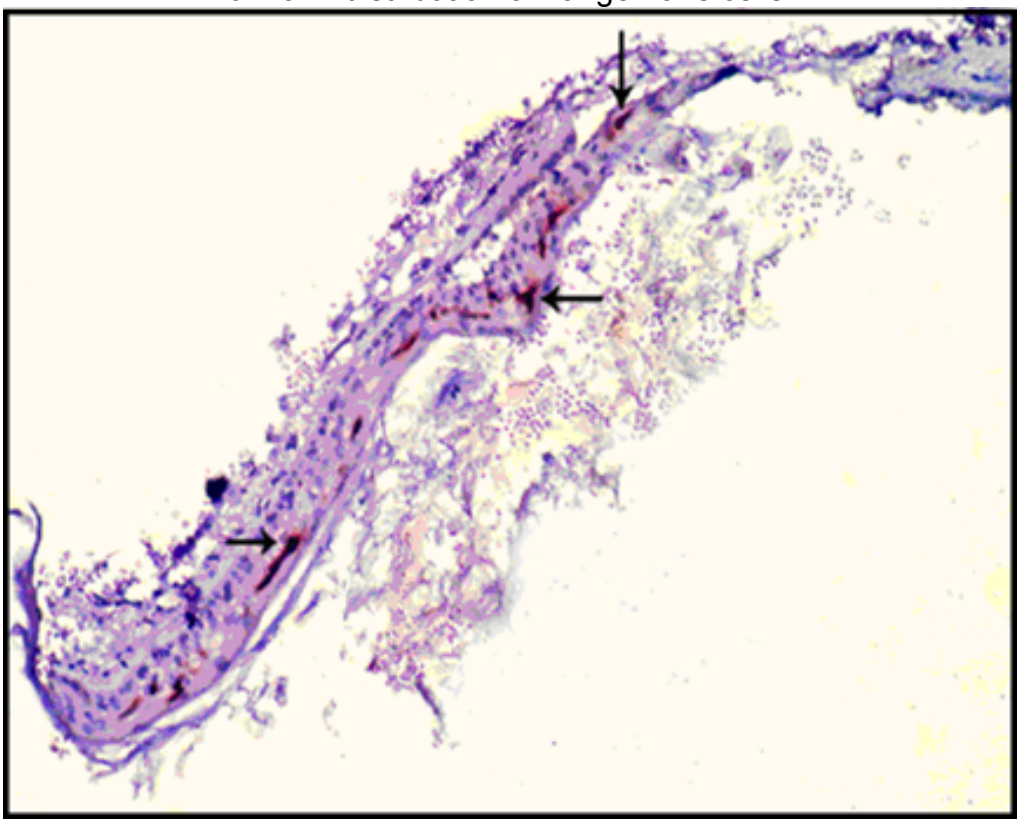


Fig. 22: Tympanic membrane in tubotympanic disease, stained with CD1a antibody, counter-stained with Harris Haematoxylin, viewed under low power. Note the increase in density of Langerhans cells when compared with the previous figure. Magnification: 183

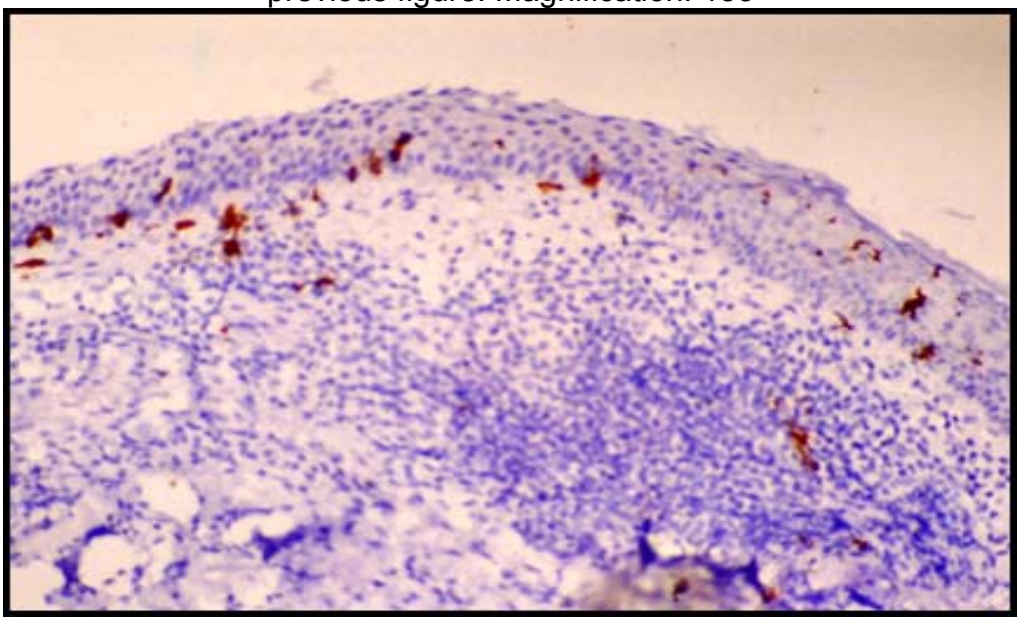
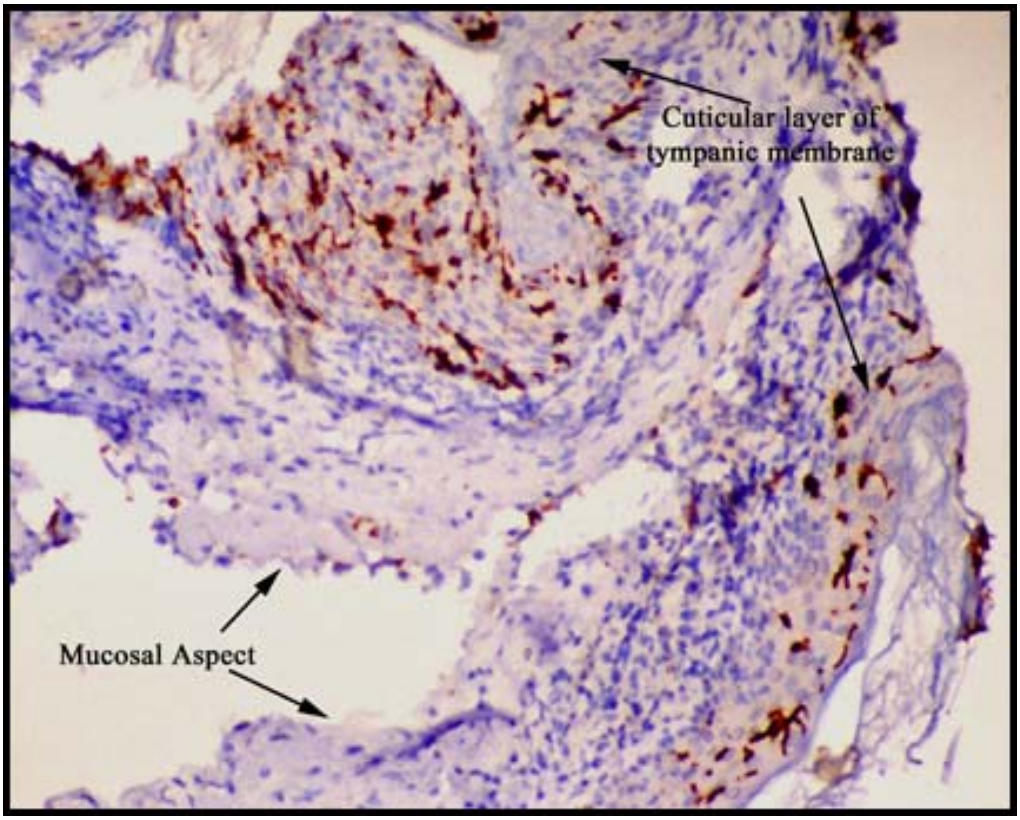


Fig. 23: Tympanic membrane in atticoantral disease, stained with CD1a antibody, counter-stained with Harris Haematoxylin. Magnification: 231. Note the obvious increase in density of Langerhans cells when compared to figures 21 and 22.



5.2. STATISTICAL ANALYSIS:

The four categories looked at were normal tympanic membrane, tympanic membrane in tubotympanic disease, tympanic membrane in atticoantral disease and tympanic membrane after traumatic perforation.

Among the four groups, number of cells per 100μm length of basement membrane, diameter of the cells, length of the processes and number of processes were studied.

Table 1

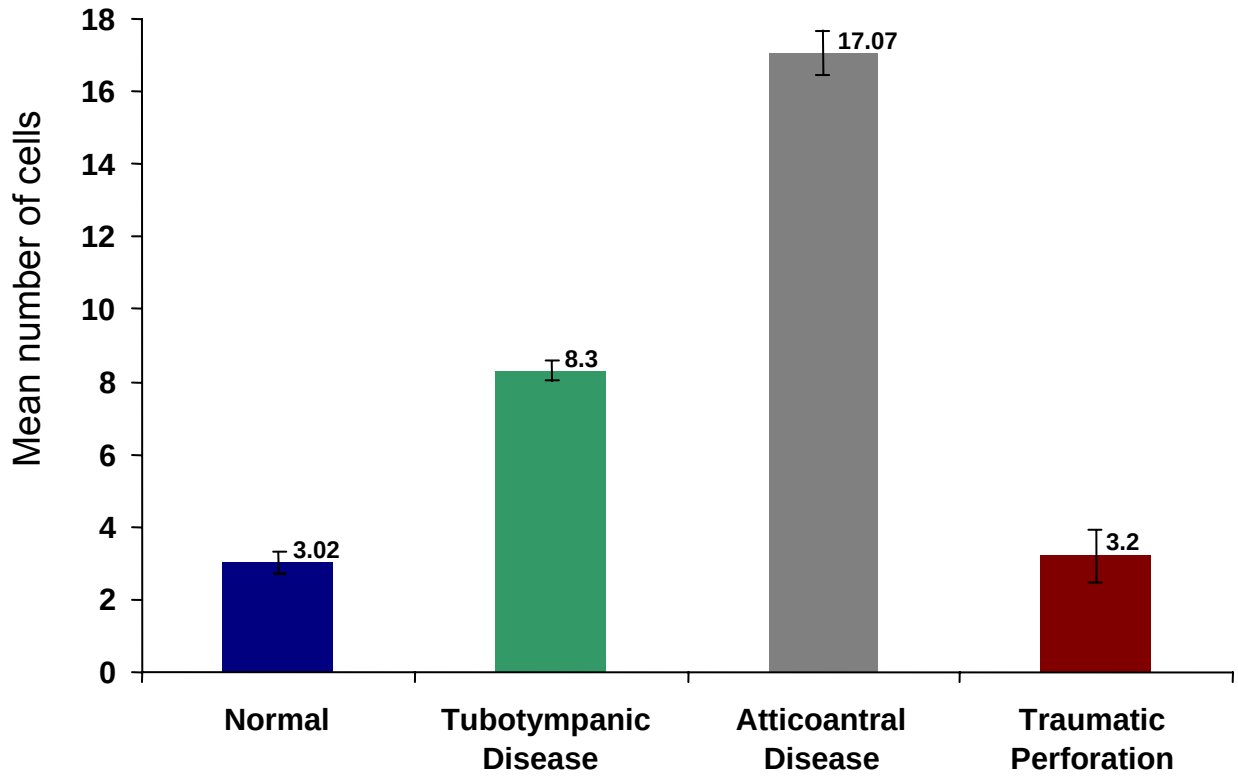
Number of cells per 100μm length of basement membrane:

Categories	Mean number	Median	Standard deviation
Normal	3.02	3.00	1.000
Tubotympanic disease	8.30	8.00	3.519
Atticoantral disease	17.07	18.00	2.637
Traumatic perforation	3.20	4.00	1.780

As is evident from Table 1 and Graph 1, the mean number of Langerhans cells seen per 100μm length of basement membrane was lowest in the normal tympanic membranes and highest in the atticoantral variety of chronic otitis media.

Graph 1

Mean number of CD1a positive cells per 100µm length of basement membrane



The mean number of cells per unit length of basement membrane when compared among the four groups, was found to be **significantly different** from each other by the Kruskal Wallis test **(p<0.001)**.

On comparing pairs of groups by the Mann Whitney U test, it was found that the mean (SD) of number of cells in tympanic membranes with tubotympanic disease, 8.3(3.5), was **significantly higher** than the mean (SD) of number of cells in the normal tympanic membrane, 3.02(1), **(p<0.001)**.

Similarly, the mean (SD) of number of cells in tympanic membranes with atticoantral disease, 17.07(2.6) was **significantly higher** than the mean (SD) of number of cells in the normal tympanic membrane, 3.02(1), **(p<0.001)**.

Interestingly, the difference between the mean number of cells in membranes with atticoantral disease, 17.07(2.6), was also significantly higher than those with tubotympanic disease, 8.3(3.5), **(p<0.001)**.

It was also seen that there was **no evidence of significance** when the mean number of cells in the normal tympanic membranes were compared with that of the traumatic perforation **(p= 0.554)**.

Inference: From these results, it can be inferred that there is a significant increase in the number of Langerhans cells in the cuticular layer of the tympanic membrane in chronic otits media of both tubotympanic and atticoantral disease. Also, the increase in atticoantral disease is significantly more than that of tubotympanic disease. However, in traumatic perforation of the tympanic membrane, there is no significant difference in the mean number of Langerhans cells.

Mean diameter of the cells

The mean diameter of each cell was calculated as the average of the horizontal and vertical diameters of the cell, horizontal diameter measured parallel to the basement membrane of the epithelium and vertical diameter measured perpendicular to it.

The range of diameters varied from 0.88μm to 3.50μm. This was important to know as the thickness of each section studied was 4μm. Thus it can assumed, that the body of a particular cell was measured only in one section and would not appear in the next.

Table 2

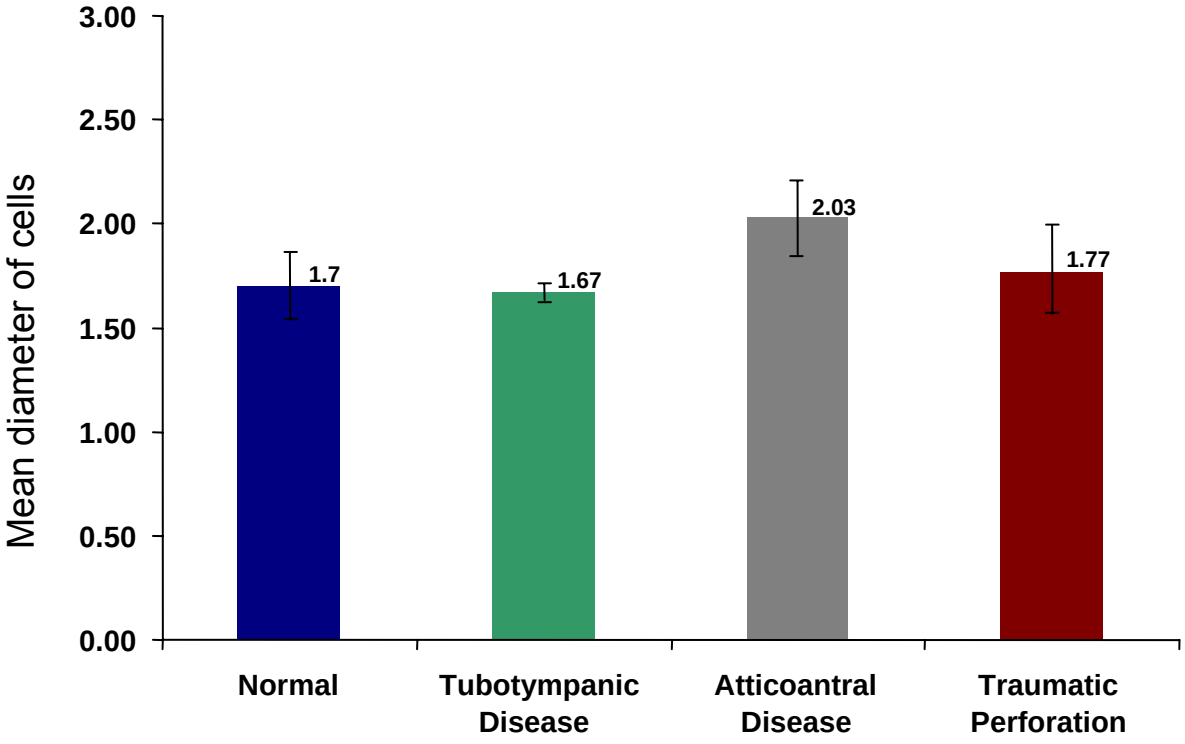
Diameter of cells

Categories	Mean (μm)	Median (μm)	Standard deviation
Normal	1.6962	1.6400	0.36421
Tubotympanic disease	1.6741	1.65000	0.32071
Atticoantral disease	2.0327	1.9800	0.51037
Traumatic perforation	1.7745	1.6700	0.34132

Table 2 and Graph 2 indicate that the mean diameter was highest in the tympanic membranes with atticoantral disease and lowest in those with tubotympanic disease.

Graph 2

Mean diameter of CD1a positive cells



The mean (SD) diameter of cells in tympanic membranes with atticoantral disease, 2.03(0.5) was **significantly higher** than that of the normal tympanic membranes, 1.7(0.36), **(p value= 0.014)**.

The mean (SD) diameter of cells in tympanic membranes with atticoantral disease, 2.03(0.5) was **significantly higher** than that of tubotympanic disease, 1.67 (0.32), **(p value<0.001)**.

Interestingly, the difference in diameters between normal tympanic membrane and tubotympanic disease was **not found to be statistically significant (p value= 0.926)** and neither was the difference between normal tympanic membrane and that with traumatic perforation **(p value= 0.559)**.

Inference: From the above results it can be inferred that there is a significant increase in the diameter of cells in atticoantral disease, but not in tubotympanic disease.

Number and length of dendritic processes

The number of processes varied from 1 to 6. For each cell, the mean length of dendritic processes was also calculated. The mean length of processes varied from 0.10μm to 2.67μm. However when the length of each process was looked at individually, the longest process measured was found to be 6.2μm. This has great significance in the interpretation of this set of results, as the thickness of the sections studied was only 4μm each. Thus, it is very likely that many of the processes measured, extended from one section to the next and hence, the entire length of every process was not measured.

Table 3

Length of dendritic processes

Category	Mean length (μm)	Median (μm)	Standard deviation
Normal	0.8815	0.7367	0.47732
Tubotympanic disease	0.8042	0.7208	0.44597
Atticoantral disease	0.8376	0.7417	0.55917
Traumatic perforation	1.0208	0.9200	0.41011

Table 3 and Graph 3 demonstrate the mean length of dendritic processes in each category. There was **no evidence of statistical significance** seen in the mean length of processes between the groups.

Table 4

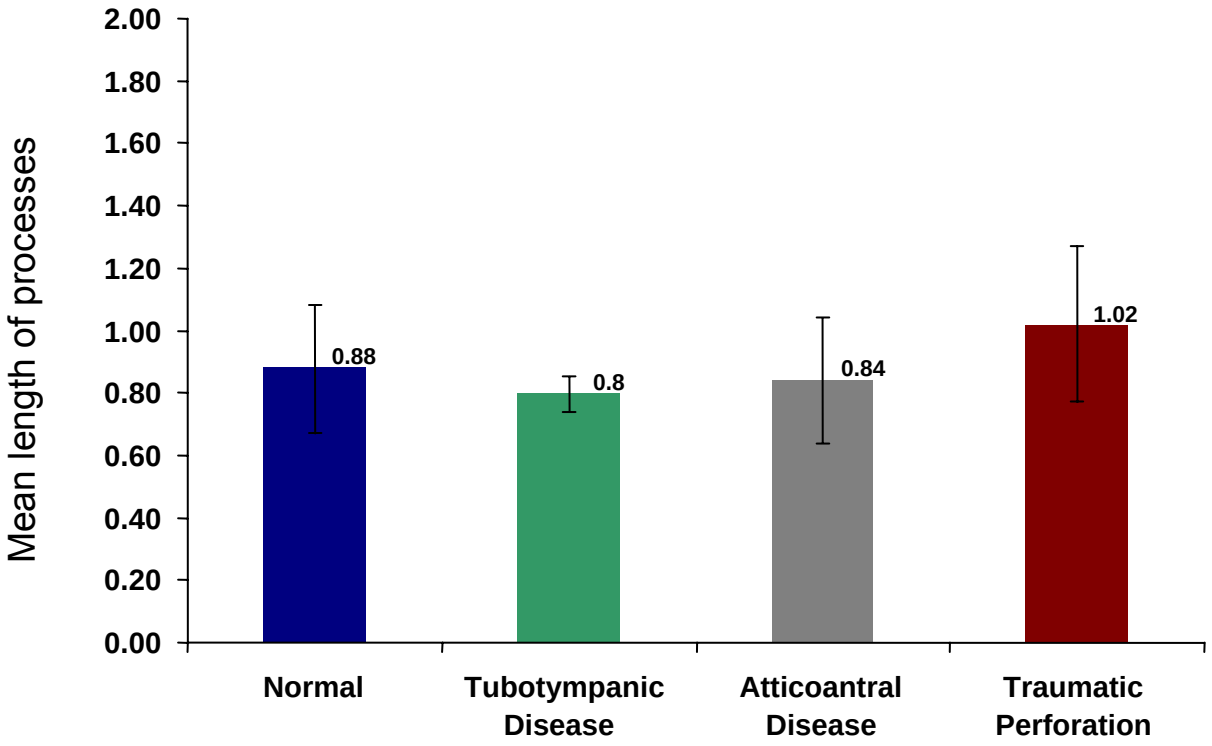
Number of dendritic processes

Category	Mean number	Median	Standard deviation
Normal	2.8500	3.00	0.93330
Tubotympanic disease	2.6308	3.00	1.07365
Atticoantral disease	2.4828	2.00	0.94946
Traumatic perforation	3.0000	3.00	0.66667

Table 4 and Graph 4 show the mean number of processes in each group studied. There was **no evidence of significance** between the 4 groups.

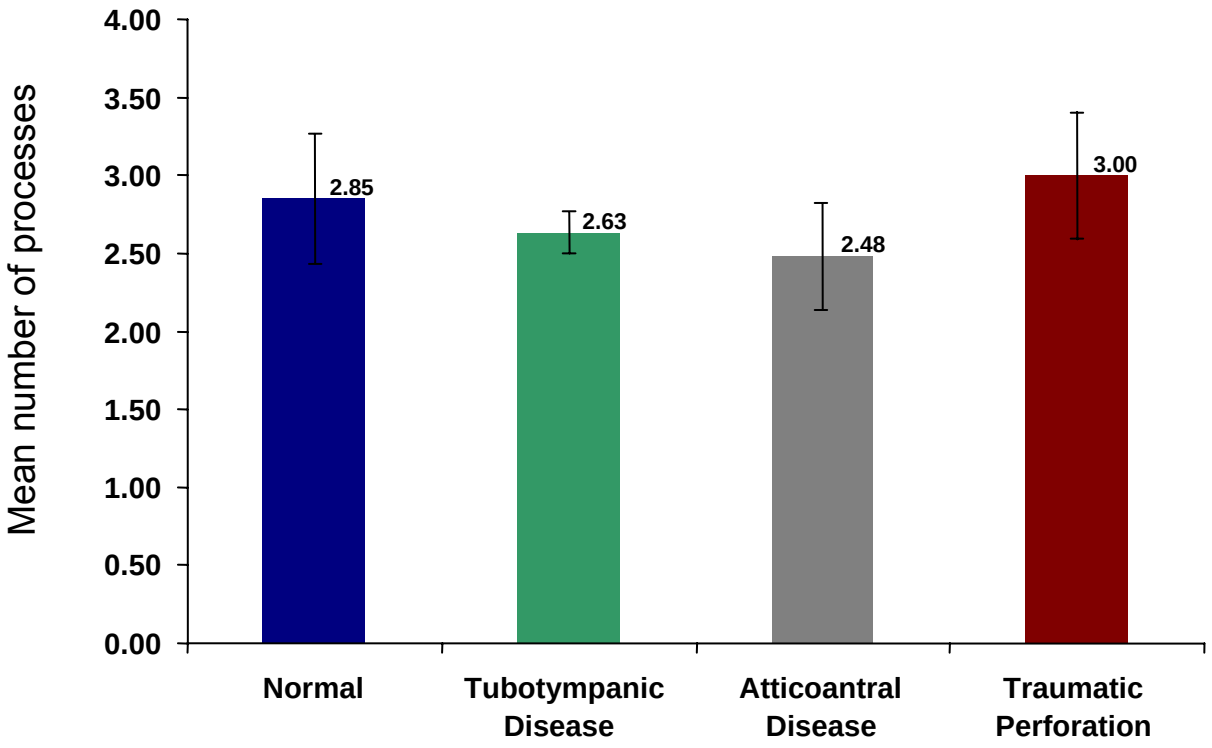
Graph 3

Mean length of dendritic processes



Graph 4

Mean number of dendritic processes



DISCUSSION

6. DISCUSSION

The Langerhans cell is part of an extremely dynamic population of dendritic cells that possess the ability to traffic tissues and migrate within the tympanic membrane. They appear to recognize antigens and present them to T lymphocytes, and possibly macrophages.

Langerhans cells have previously been described in the tympanic membranes of patients with chronic otitis media[138-140] and in the matrix of cholesteatomas.[40, 73, 134-137] However, the presence and distribution of these cells in the normal tympanic membrane are controversial. Also, although it has been postulated that these cells play a role in the pathogenesis of chronic otitis media,[40, 58, 59, 73, 134, 138,] specifically of cholesteatoma, a quantification of the increase in the number of these cells in these conditions has not been attempted.

This is the first study that quantifies the distribution of Langerhans cells in the normal tympanic membrane, and quantifies their increase in the tympanic membrane in chronic otitis media of the tubotympanic and atticoantral varieties.

A number of techniques have been described for the identification of Langerhans cells by light microscopy.[31, 59, 69-74] These techniques present problems with the specificity of reaction and complication of procedure. Of these, the tests for cell surface markers are most useful. Studies by Palva and Taskinen[59] and Harrist et al[80] showed that CD1a is a better marker for Langerhans cells than others, with regard to sensitivity and specificity for the cell. A positive staining reaction with CD1a (also called OKT6/Leu6) is considered by

some as the **gold standard** for Langerhans cell identification, despite the fact that this epitope is also present in the cortical thymocyte because, with maturation, cortical thymocytes lose CD1a before exiting the thymus.[45, 88] Thus for this study, staining for the cell surface antigen CD1a, was employed.

The immunological role of the Langerhans cell is now undisputed. Also, their increase[60,88, 106-111] and decrease[112-119] in number in various disease conditions have been previously noted. This study looks to quantify their increase in the tympanic membrane in chronic otitis media.

The first part of this study was to verify the presence of these cells in the normal tympanic membrane. Looking at previous work done on the normal tympanic membrane, some important questions may be raised. Veldman[40] stated that the normal tympanic membrane was probably devoid of Langerhans cells. Hussl et al[37] and Gantz[122] performed studies on the normal human tympanic membrane and these studies had contrasting findings.

Hussl et al[37] looked a cadaveric tympanic membranes from patients who had died of causes other than ear disease and had had no history of middle ear diseases, removed 14-21 hours after death. He found cells in the epidermal layer of the tympanic membrane which were HLA-DR antigen positive but negative for CD1a. However, he did not quantify the distribution of these cells. The explanation given for this was that possibly cell surface antigens like CD1a disintegrate postmortem.

In the present study however, CD1a positive Langerhans cells are seen in both biopsies of normal tympanic membrane studied. Both these membrane were collected between 24 and 48 hours postmortem.

Ichimiya et al[133] studied normal tympanic membrane in mice. He found that CD1a positive cells were absent in the normal tympanic membrane except in the pars flaccida, and in the manubrial and annular parts of the pars tensa. The rest of the pars tensa was devoid of Langerhans cells. Gantz[122] looked at epidermal Langerhans cells in cholesteatoma matrix and compared them with normal tympanic membrane and normal ear canal skin. He felt that the cells were present in the normal tympanic membrane, although in lower numbers than in the cholesteatoma matrix. His finding was that Langerhans cells were evenly distributed through the entire surface of the tympanic membrane and not limited to the pars flaccida and the annular and manubrial regions of the pars tensa

In our study, we agree with the findings of Gantz[122] that the pars tensa of the human tympanic membrane uniformly shows the presence of CD1a positive Langerhans cells and not with those of Ichimiya et al[133] who stated that the Langerhans cells were limited to the pars flaccida and in the manubrial and annular parts of the pars tensa. Gantz, however, failed to quantify the distribution of cells in order to establish their increase in chronic otitis media. Our study, on the other hand proceeds to do that.

The unavailability of fresh tympanic membrane tissue makes studies on the normal tympanic membrane difficult. The normal tympanic membrane is almost never removed as a routine part of surgical procedures. Therefore, the

only means of collection of the tissue was from freshly donated cadavers to the Department of Anatomy, donation being done within 48 hours of death. Only 2 such biopsies could be obtained. This low number of normal tympanic membranes studied is a draw back in this study.

Most investigators of Langerhans cells in chronic otitis media studied either the middle ear mucosa[138-140] in these conditions or the matrix of the cholesteatoma in atticoantral disease. [40, 73, 134-137] This study, however, compares numbers of Langerhans cells in the tympanic membrane in these conditions. It was found that there is a statistically significant increase in the number of Langerhans cells in chronic otitis media of both the tubotympanic and atticoantral varieties when compared with the normal tympanic membrane. Interestingly, the difference between the mean number of cells in tubotympanic and atticoantral disease was also statistically significant. These findings support the theory of Veldman[40] and van Dijk et al[138] that these cells have an important role in the pathogenesis of these diseases.

Sections show these cells concentrated within the stratified squamous epithelium but also in the subepithelial connective tissue and in close conjunction with lymphoid aggregates. This supports the migratory role of these Langerhans cells.

Langerhans calls have been known to control the lymphoid cell traffic.[59] Our frequent finding of these cells in the subepithelial follicles is in accordance with this function.

Park[58] reported that the matrix of cholesteatoma had Langerhans cells whose processes were longer and more branched. However, her did not report an increase in cell diameter. An interesting finding of this study is that the mean diameter of cells is increased significantly in the tympanic membranes of cases with atticoantral disease when compared with the normal tympanic membranes. This increase in cell size is probably a result of their immunological activation in the presence of antigen. However, the cell diameter does not significantly increase in tubotympanic disease.

Veldman et al,[138] Gantz[122] and Takahashi and Nakano,[73] all suggested that Langerhans cells within the cholesteatoma matrix were responsible for generating and maintaining the chronic immunological reactions of this disease. However, Kaehoene et al[139] and Palva and Taskinen[59] did not support this hypothesis. They felt that the presence of Langerhans cells in the secretory epithelium of subjects with secretory otitis media and chronic otitis media, in which there was not the slightest sign of keratinisation argued against the specific role for Langerhans cells in recurrent cholesteatoma. They felt that Langerhans cells should simply be viewed as normal defense cells.

Our finding of significant increase in the number of Langerhans cells in the tubotympanic variety of chronic otitis media is in favour of the latter theory. However, their significant increase in chronic otitis media of atticoantral variety suggests that these cells may be involved in the proliferation of existing cholesteatoma.

Contrary to the observation made by Park[58] that Langerhans cells found in the matrix of cholesteatoma had longer and increased number of dendritic processes, we found that the difference in the number and length of dendritic processes between the groups was not statistically significant. One possible explanation could be that adequate samples of atticoantral disease and normal tympanic membrane were not studied. The other possibility is that this increase in number of processes and arborisation occurs only in the cholesteatoma matrix and not in the tympanic membrane.

Takahashi and Nakano[73] reported that in the cholesteatomatous tissue, dendritic processes were mainly directed towards the surface of the epithelium. In the present study, although this was noted in some areas, it was not a universal feature. This can be explained by the fact that the processes inter-communicate greatly with those of other dendritic cells, passing on antigenic information between them and also with lymphocytes.

One of the diseased biopsies obtained was from the margins of a traumatic perforation of the tympanic membrane. When the cells properties and distribution in this specimen was compared with the other groups, it was seen that there was no significant difference in the number of cells per unit length of basement membrane, diameter of cells, length of processes and number of processes between this biopsy and the normal tympanic membrane. This supports the role of the Langerhans cell in antigen presentation as this biopsy was devoid of infection.

Clinical Relevance:

Interference with Langerhans cell-Tcell function is a goal of future research. Inactivation of cell receptors or removal of Langerhans cells from the skin is probably the key to influencing the clinical behaviour of various skin related diseases including cholesteatoma.

Through immunopathologic evaluation, the clinical aggressiveness of a cholesteatoma could become predictable, having consequences for the future handling of cholesteatomas.

The knowledge of the role of Langerhans cells in the pathogenesis of cholesteatoma is especially relevant, as medical manipulation of Langerhans cells could be used as an adjuvant to surgery in the future handling of cholesteatomas.

CONCLUSIONS

7. CONCLUSIONS

1. The normal tympanic membrane does contain Langerhans cells distributed through the pars tensa.

2. The mean number of Langerhans cells per unit length of basement membrane in chronic otitis media of the tubotympanic variety is significantly higher than that of the normal tympanic membrane.

3. The mean number of Langerhans cells per unit length of basement membrane in chronic otitis media of the atticoantral variety is significantly higher than that of the normal tympanic membrane and chronic otitis media of the tubotympanic variety.

4. In chronic otitis media of the atticoantral variety, the mean diameter of the cells increases significantly.

5. The mean number of dendritic processes and their length do not increase significantly in chronic otitis media.

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